EXPERIMENTS ON THE VISIBILITY OF THE POLYHEDRAL VIRUSES.

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In recent years the conception of the existence of filterable viruses as excitants of disease has been considerably extended.[1] It is now generally recognized that these agents, whatever they may be, operate in plants and insects as well as in the higher animals and man. But they are very elusive and it is questionable whether they may be seen microscopically; some of them most probably not. We have concentrated on this question of visibility with what we believe to be unusually favorable material, namely, the polyhedral viruses of insects, and in particular those which produce a "wilt disease" of gypsy larvæ and "grasserie" of silkworms. It has been claimed that these diseases are caused by a chlamydozoan[2,3] and by a *Borrellina*.[4] Previous work by one of us revealed no organisms of etiological significance in either.[5,6] We wish now to report briefly our comparison of diseased and normal blood made because the viruses are present in the former and absent in the latter.

Technique.

We employed many methods of fixation and staining and tried to devise new ones without finding a technique more satisfactory than

[1] Rivers, T. M., *J. Bact.*, 1927, xiv, 217.
[2] Wolff, M., *Mitt. Kaiser Wilhelm Inst. Landes. Bromberg*, 1910, iii, 69.
[3] von Prowazek, S., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxvii, 268.
[4] Paillot, A., *Ann. Inst. Pasteur*, 1926, xl, 314.
[5] Glaser, R. W., *J. Agric. Research*, 1915, iv, 101.
[6] Glaser, R. W., *Ann. Entomol. Soc. America*, 1927, xx, 319.

that of Giemsa. Various methods of vital staining were attempted but shed no light on the problem. Our optical equipment consisted of one of the latest Zeiss microscopes with lens combinations giving magnifications of from 900 to 2,400 diameters. In the examination of fresh preparations with different types of condensers a strong arc light was found indispensable. The condensers most frequently used were the paraboloid condenser which shows the reflections of colloidal particles down to 100 mμ in diameter, and the cardioid condenser (also called the cardioid ultramicroscope) which reveals the reflections of particles down to 50 mμ. The ultramicroscope of Siedentopf and Zsigmondy by which one may detect reflections of particles as small as 10–15 mμ was also employed.[7]

Qualitative Comparisons.

A representative protocol follows.

Blood from early cases of gipsy moth wilt and blood from early cases of silkworm grasserie were separately collected under sterile conditions. The blood from each insect was divided into two portions. With one of these from each diseased gipsy larva, five healthy gipsy larvæ were inoculated; and with one from each diseased silkworm, five healthy silkworms were likewise injected. The healthy gipsy larvæ were collected from a lightly infested tract of woods and examined for the presence or absence of polyhedral bodies according to the well known blood method. Only those larvæ entirely free from polyhedra were used. The silkworms which we employed had been reared in the laboratory, and since the entire stock was free from grasserie, no further precaution was necessary. All the inoculated insects subsequently died of either wilt or grasserie within the expected time, showing that the blood contained the infectious agent. Controls, inoculated with blood inactivated by heat, survived. The other portion of the infectious blood from each animal was studied immediately after being drawn in both the fresh and the fixed and stained condition.

The methods of Romanowsky, of Wright, and especially of Giemsa very often brought to light, in exceedingly thin films of the diseased blood, minute single or double coccoidal bodies colored pale pink or blue. These bodies could be best seen with a 1.5 mm. apochromatic

[7] International Critical Tables, New York, 1926, i, $\mu = \frac{1}{1,000}$ mm.; mμ = $\frac{1}{1,000,000}$ mm.

objective, and a 10 or 20 ocular. Beside these little stained particles nothing else was visible except blood cells, unstained polyhedra, and some debris. But when normal blood was prepared and studied in an identical manner the same type of stained particles was observed. Examination with the paraboloid or cardioid condensers of fixed and stained normal and diseased blood showed many minute particles of various sizes not revealed by ordinary illumination. These were not dye particles, because Giemsa's fluid is not colloidal and the formation of precipitates was avoided. With fresh material no evidence of independent motility of the particles was ever observed when care was taken to exclude air currents from the preparations, and when vibrations were reduced to a minimum. As the diseases progressed, and the tissues commenced to disintegrate, the particles in the blood increased numerically. Up to a certain point the condition then obtaining could be imitated in normal blood by permitting it to stand for a few days until the cells disintegrated and liberated much particulate matter. Heavily diseased blood, tissues, and dead larvæ were, however, useless for finer microscopic study, owing to the rapid final lysis of all the tissues with the discharge of protein particles, fat globules, pigment granules, urates, and various other substances.

From such comparisons made between normal fresh blood and infectious fresh blood, and between normal stained blood and infectious stained blood, we have come to the conclusion that no observable qualitative distinction exists between the various particles found in the normal and in the diseased condition in either wilt disease or grasserie, and further that none of these particles resemble minute cells or organisms.

Quantitative Comparisons.

If some of the particles within the diseased insects are etiologically important, they should numerically outrank similar particles found within the normal individuals, and for this reason we attempted to secure quantitative data. That such may be of value is indicated by the fact that, in most bacterial and other infections of insects, the living causative agents are usually very numerous. This is probably due to the open form of circulation. Paillot[8] points out that diseases

[8] Paillot, A., *Ann. Epiphyties*, 1922, viii, 265.

in insects caused by a limited number of microorganisms, which produce injury through soluble toxins, are as yet unknown. Many counts were accordingly made of small microscopic and ultramicroscopic particles in normal blood and in blood from early cases of grasserie. We present a protocol of one experiment out of twelve.

TABLE I.

Counts of Particles in Silkworm Blood Made with the Aid of a Cardioid Condenser.

The blood from three normal and three grasserie-infected silkworms was used. Two preparations were made from each worm and the particles in ten fields of every specimen were enumerated. Lens combination: 1.5 mm. objective, and 10 ocular.

Worm	Preparation	1	2	3	4	5	6	7	8	9	10
Grasserie blood.											
A	1	165	154	83	95	115	43	195	29	42	58
	2	10	22	35	75	28	45				
B	1	24	67	68	76	55	74	22	26	52	32
	2	8	6	9	11	8	2	9	11	4	7
C	1	33	40	22	13	21	36	45	20	75	21
	2	18	26	30	43	40	35	21	9	42	48
Normal blood.											
A′	1	160	45	39	55	53	22	89	41	27	46
	2	80	51	110	60	35	75	70	19	44	38
B′	1	8	30	50	20	25	30	15	53	88	24
	2	14	7	25	38	37	2	45	40	8	56
C′	1	26	14	52	19	30	63	21	47	17	35
	2	43	7	56	50	20	23	38	29	24	25

0.1 cc. of normal silkworm blood and 0.1 cc. of blood from a silkworm infected with grasserie by inoculation 4 days previously were each diluted 100 times with sterile, distilled water. The diluted blood was then centrifuged ½ hour at 1,000 R.P.M. to eliminate the blood cells, the larger particles, and the polyhedra. It was found in 1927,[6] that centrifuging the virus for 4 hours at 2,000 R.P.M. did not reduce its infectivity. 1 standard drop (0.1 cc.) of the clear upper fluid from each lot was then placed on the center of each of a series of scrupulously clean cover-slips.

These were floated over mercury and dried in air, to obtain a uniform distribution of the particles present. A diaphragm was inserted into the ocular to reduce to about one-half the field of vision and the particles were counted both microscopically, and ultramicroscopically (with the aid of the cardioid condenser). This was done for ten fields on each cover-slip. In each of the twelve experiments two cover-slips representing normal blood and two representing grasserie blood were used. The microscopic shapes of the particles and their ultramicroscopic reflections seemed identical as studied in normal and diseased blood, and the size varied from about 50–100 mμ to approximately 0.5μ depending upon the type of illumination employed.

The average microscopic particle counts of diseased and normal blood were naturally considerably lower, owing to lesser visibility, than the average ultramicroscopic particle counts in the same preparations. It is significant, however, that the same ratio was found between the number of particles in the diseased and normal blood when examined in these two ways.

A statistical analysis[9] of the determinations (Table I) shows that the average ultramicroscopic particle count for grasserie blood was 42.3 $\pm$ 3.3, and for normal blood, 40.2 $\pm$ 2.3. The standard deviation for grasserie blood was calculated to be 38.3 $\pm$ 2.4, and for normal blood, 26.8 $\pm$ 1.7. The difference in the variability was 11.4 $\pm$ 2.9, or 3.9 times the probable error. It seems, therefore, that there is no outspoken difference in the number of ultramicroscopically visible particles existing in normal and grasserie-infected blood, although the number in the latter is slightly the more variable as one would expect in the case of animals whose tissues are undergoing such rapid degeneration.

SUMMARY.

With the techniques employed we have not been able to detect any qualitative differences between the particles visible in normal blood and in blood from cases of wilt disease and grasserie. Of the two conditions we can speak more definitely in the case of grasserie, because this we have studied quantitatively as well, that is to say, the particles visible microscopically and ultramicroscopically have been counted without bringing to light any marked difference between

[9] Kindly made by Dr. John W. Gowen.

normal and diseased blood. This leads us to believe that the virus of wilt disease is probably invisible, and the virus of grasserie almost certainly so, when studied with the optical equipment that we have used, and that further evidence will be necessary before one can accept the chlamydozoa or the *Borrellina* as the active etiological agents. Although these two polyhedral viruses do not appear to be visibly particulate, it does not follow that other filterable viruses are not organized in this way. Each should be considered on its own merits.

EFFECT OF FORMALIN ON THE VIRUS OF VESICULAR STOMATITIS OF HORSES.

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From time to time reports have appeared dealing with active immunization with filtrable or allied viruses that have been killed by weak solutions of formalin.

Among those recently making favorable reports are Cumming,[1] Vallée, Carré and Rinjard,[2] the British Foot-and-Mouth Disease Research Committee,[3] and Hunt and Falk.[4] Cumming worked with rabies virus treated less than 24 hours with 0.4 per cent formaldehyde, while Vallée and his coworkers studied foot-and-mouth disease virus exposed to 0.5 per cent formalin at 20°C. for from 4 to 7 days. The British Committee used the formalin in 0.1 per cent strength, which was added to filtered contents of foot-and-mouth disease vesicles, the mixture being kept for 48 hours at 26°C. before use. Hunt and Falk tested vaccinia material to which was added formaldehyde in 0.1 per cent concentration for 12 hours, the mixture being kept at unstated temperature. Besides these favorable reports, failures have been recorded. Thus Abramson and Gerber[5] were unable to secure immunization with formalinized virus of poliomyelitis.

It is obvious that the subject is one of wide interest and importance, for if active immunization can be secured with filtrable viruses, altered by this simple chemical means so that they are incapable of inducing specific lesions, a great step forward will have been taken. Up to the present, active immunity has been secured only by the use of attenuated or modified, but still living viruses, capable of producing some, although at times very slight, specific changes in the tissues.

[1] Cumming, J. G., *J. Infect. Dis.*, 1914, xiv, 33.

[2] Vallée, H., Carré, H., and Rinjard, P., *Rec. méd. vét.*, 1925, ci, 297; *Rev. gén. méd. vét.*, 1926, xxxv, 129.

[3] Second Progress Report of the Foot-and-Mouth Disease Research Committee, Ministry of Agriculture and Fisheries, London, 1927, 1–117.

[4] Hunt, L. W., and Falk, I. S., *J. Immunol.*, 1927, xiv, 347.

[5] Abramson, H. L., and Gerber, H., *J. Immunol.*, 1918, iii, 435.

Our study was made with a strain of the virus of vesicular stomatitis of horses, already described,[6,7] which has been shown to be closely related to the virus of foot-and-mouth disease. It was designed to determine (*a*) whether protection could be obtained with formalinized virus, and (*b*) whether the protection could be construed as arising from the effects of a killed antigen.

Before proceeding with the description of experiments, we wish to recall some earlier conclusions derived from tests with antiseptics on the virus of foot-and-mouth disease.[8] The remarkable resistance of the latter virus to certain chemicals was found to be due to the fact that the reagents coagulate the proteins of the medium in which the virus is ordinarily distributed. As a result of this coagulation, the positive charge of the virus, and its minute size, the active material is held within large coagula and prevented from coming into immediate contact with the chemicals. Similar conditions have been found to occur in the instance of the virus of vesicular stomatitis,[7] in which the virus proved resistant to many chemicals which coagulate proteins. Furthermore, one of us (Long) has found that still another chemical, namely, neutral acriflavine in 1 per cent solution, acts as a coagulant so that the virus proves resistant when submitted to it for at least 24 hours. Since formalin is a substance which coagulates proteins, we studied the virucidal action by means of a series of controls, as shown in the following tests.

Viability of Formalinized Virus.

In the preparation of formalinized virus of vesicular stomatitis, we followed mainly the methods employed by the British Foot-and-Mouth Disease Research Committee.[3] The Committee have studied the problem in connection with the virus of foot-and-mouth disease more extensively than any other recent workers.

[6] Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx, 147.

[7] Olitsky, P. K., *J. Exp. Med.*, 1927, xlv, 969.

[8] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 815; and the Report of the American Commission to Study Foot-and-Mouth Disease, to be published by the U. S. Bureau of Animal Industry.

Preparation.—The virus consisted of the clear fluid derived from vesicles in the pads of from three to over ten guinea pigs injected 24 hours previously, as a rule, with guinea pig passage virus of vesicular stomatitis. The fluid was aspirated into a syringe and diluted 1 in 50 with phosphate buffer solution at a pH of 7.5 or 7.6. The diluted material was filtered through a Berkefeld V candle and the major portion of the filtrate was used for formalinization and the remainder for control tests of pathogenicity in guinea pigs. Six lots of filtered virus were prepared at different times. To four, 0.1 per cent neutral formalin was added, to one, 0.5 per cent, and to the sixth, 10 per cent. The formalinized virus was kept in an incubator at 26°C. for 48 hours or longer, and then tested by injection into the pads of guinea pigs.

The 0.5 and 10 per cent formalinized material proved too injurious for use: extensive necrosis (or "wet" gangrene) of the pad followed the use of the 10 per cent mixture, and a somewhat less degree of destruction, the 0.5 per cent mixture. Although the virus was killed in both instances after 2 days at 26°C., the factors of injury, confusion of specific and non-specific effects, and mortality led us to abandon the higher concentrations of formalin in favor of 0.1 per cent of this chemical, which is the concentration generally employed in preparing the so called "vaccines" of filtrable viruses. As will be shown later, even 0.1 per cent formalin is not wholly free from injurious effects—a mild edema being sometimes induced in guinea pigs. Similar effects in rabbits were observed by Hunt and Falk.[4]

Titration of Filtered Virus.—In order to determine the activity of the original filtered virus before utilizing mixtures of it with formalin for immunization tests, it was essential to show that the specimens of virus employed produced the characteristic lesion in guinea pig pads. Of three samples accurately titrated, one induced experimental vesicular stomatitis in a 1:1,000,000 dilution, another in 1:500,000, and a third in 1:100,000 dilution. The remaining samples were injected, as a routine procedure, in dilutions of 1:100 or 1:1,000, all being capable at these dilutions of producing typical lesions.

From now on we shall designate as "vaccine" the 0.1 per cent formalinized virus. We wish again to emphasize the fact already mentioned that this strength of formalin has in some animals an injurious action on the pad tissues, as shown by edema following injection. This injurious action is uninfluenced by either strong or weak virus; hence it must be ascribed to the chemical alone.

Living Virus in the Vaccine.

Tests were first made for evidence of living virus in the mixtures with formalin. After the freshly made mixtures had stood 48 hours at 26°C., 0.5 cc. was injected into each posterior pad of at least two guinea pigs. The results were as follows:

Vaccine 2.—Two guinea pigs, A and B, injected. After 4 days, A showed a single vesicle on one pad, yielding clear, serous fluid. B exhibited a similar condi-

tion on the 3rd day. Guinea Pig B was etherized, the affected pad removed and ground in buffer solution with sterile sand. The pad emulsion was injected into the pads of two normal animals. Both developed typical vesicular lesions on the following day.

Vaccine 5.—Two guinea pigs, A and B, injected. On the next day, A showed a single vesicle on the right pad, while B was unaffected. 14 days after the original inoculation, B was injected in both pads with active virus. The following day, typical vesicles had appeared on both pads.

Vaccine 6.—Four guinea pigs, A, B, C, and *D*, injected. 24 to 48 hours later, all showed either single vesicles or no visible lesions; yet on pricking the pad with a needle, clear, serous fluid exuded. This clear fluid from Guinea Pig D was inoculated into the pads of Guinea Pig E, in which typical vesicles arose within 24 hours. Guinea Pigs A, B, and C were reinjected with active virus after 14 days, and as none responded they can be regarded as immune, a finding which confirmed our opinion that the initial lesions following the injection of vaccine were those of vesicular stomatitis.

Vaccine 7.—Three guinea pigs, A, B, and C, injected. After 24 to 48 hours, no visible lesions appeared, except edema. On pricking the pads with a needle, a clear, serous fluid exuded. Guinea Pig A was etherized, the pads removed, ground in buffer solution with sand, and the emulsion inoculated into the pads of a normal animal. The latter developed typical vesicles by the next day.

From the above four tests, it may be concluded that the so called vaccines as prepared with 0.1 per cent formalin contain living virus. In contrast, we wish to record that guinea pigs in which pad inoculation was made with 0.5 per cent and 10 per cent formalinized virus developed severe necrotic lesions, from the fluid contents of which the virus of vesicular stomatitis could not be recovered. One may infer that the virus is killed by these concentrations.

The experiments described indicate that the usual concentration of formalin (0.1 per cent) employed for the production of so called vaccines of filtrable viruses does not kill the virus of vesicular stomatitis after a contact of at least 2 days at 26°C. The fact should, however, be noted that the lesions induced by the treated virus are generally slight, and that they may be confused with edema resulting from the action of the formalin alone. Simple inspection of the inoculated site is not sufficient to determine whether the effect is wholly chemical or due in part to living virus. In order to determine this, one should prick the superficial skin with hypodermic needles so as to cause any existing fluid to exude, which fluid, or that obtained from the emulsi-

fied pad, should be reinoculated into normal animals and the effects observed. Finally, recovered guinea pigs should be subjected to a later injection of active virus to determine if immunity exists,[9] and any pads showing suspicious lesions should be examined histologically for the presence of characteristic pathological changes and of the inclusion bodies which we have already described.[10] Such has been our own routine procedure.

Since, as the tests described above show, 0.1 per cent formalin fails to kill the vesicular stomatitis virus after 48 hours contact, we next studied the point at which the infectiousness of the so called vaccine ceased.

Vaccine 7 (0.1 Per Cent Formalin.)—Three guinea pigs injected with this vaccine kept for 48 hours at 26°C. after its preparation, were found to harbor the virus, a fact demonstrated by the preceding protocol. Two of three animals which received the same material after it had stood for 72 hours at 26°C. also gave evidence of containing living virus, after transferring the ground pads of the two animals to normal guinea pigs, which, in turn, developed characteristic vesicles. Six guinea pigs injected with the preparation after it had stood for 96 and 120 hours at 26°C. exhibited no effects which could be attributed to living virus. These unaffected animals were reinoculated in the pads about 10 days later with active, undiluted virus. All responded promptly with characteristic vesicles.

It is plain that Vaccine 7 contained living virus for 72 hours but not for 96 hours. The fact should be noted, in addition, that a single injection into the pads of formalinized virus, unless living virus is present in it, fails to immunize the animals.

Effect of Repeated Injections of Killed Formalinized Virus.

In view of the fact that previous workers have reported the production of active immunity by means of repeated injections of formalinized vaccines in which they supposed the virus to have been killed, we studied the effect of repeated inoculations with some of our formalin-

[9] It has already been established[6,7] that in experimental vesicular stomatitis, the induction of vesicles, no matter how small or few in number, is followed by a solid resistance to undiluted active virus. The same conditions prevail in experimental foot-and-mouth disease of the guinea pig (Olitsky, P. K., Schoening, H. W., and Traum, J., *North American Vet.*, 1927, viii, 42).

[10] Olitsky, P. K., and Long, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxv, 287.

ized virus. But, as the following protocol shows, we were unable to detect any immunity in animals "vaccinated" with dead virus.

Two vaccines were selected, Nos. 2 and 3, made with 0.1 per cent formalin, which had been shown to contain living virus after 48 hours at 26°C., and which had been kept thereafter in the cold (5°C.). After 34 and 39 days respectively at this temperature, the vaccines were injected into the pads of guinea pigs. No effects of living virus could be detected by the methods already described. None of the six animals of this series proved resistant to a later injection of active virus.

The same preparations were tested again after they had been kept at 5°C. for a longer period (70 days in the case of Vaccine 2 and 75 days[11] in that of No. 3). Twelve guinea pigs were given a series of seven injections with one or the other vaccine, six receiving 0.5 cc. intramuscularly and six others 1 cc. intradermally and subcutaneously, that is, 0.5 cc. in each of the posterior pads. The first six injections were given at 3 day intervals, and the seventh 4 days after the sixth. 3 days after the injections had been completed, the ten surviving animals were inoculated into the pads with virus of known activity. All responded within 24 hours with characteristic vesicles.

In the following experiment it will be shown that it is possible to induce resistance to active, undiluted virus by a single intramuscular or intracutaneous injection of living, untreated virus, although under these conditions no demonstrable lesions occur.

Fluid from vesicles 24 hours old of guinea pigs inoculated with passage virus, whether diluted 1:10 or undiluted, induced no demonstrable lesions when injected intracutaneously into the shaved skin of the abdomen, or intramuscularly into the thigh. In four of the seven animals thus treated, the pads were scratched, though not inoculated, at the time of the virus injection. These pads developed no lesions.[12] All the guinea pigs resisted pad injection of undiluted, active virus from 10 to 23 days later.

The virus of vesicular stomatitis, therefore, appears to behave as do some other filtrable viruses, that is, immunity can be induced only when living virus is employed.

[11] The experimenters of the British Committee state that the activity of their killed foot-and-mouth disease vaccines persists in the cold for at least 197days, and that protection is first noted 48 hours after injection.

[12] Absence of localization in the injured pad may be ascribed in these instances to the fact that the strain of virus which we used had lost its original feeble power to induce secondary lesions. The failure of the vesicular stomatitis virus to induce demonstrable lesions after intramuscular or intracutaneous injection was first noted in cattle.[6]

SUMMARY AND CONCLUSIONS.

The virus of vesicular stomatitis is not readily killed by formalin. This chemical is one of a group which coagulates the proteins of the medium in which the virus is usually contained. It has already been found[7] that other reagents of the protein-coagulating group are not actively virucidal, and the effect of formalin in this instance is therefore characteristic of the group.

The so called formalinized vaccines which give rise to immunity can be shown to have done so because of the presence of living virus. A single injection of such so called "vaccine," or of other material containing living virus, is capable of inducing immunity in guinea pigs. No protection, however, follows a single injection of dead virus. Furthermore, repeated inoculations of virus killed by formalin likewise fail to induce resistance against subsequent injections of the living virus.

It is concluded, with respect to the virus of vesicular stomatitis, that the use of formalin has failed to solve the problem of active immunization with dead virus.

THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

IV. Anaphylaxis with Two Non-Type-Specific Fractions.

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Anaphylactic experiments with bacterial proteins have presented more difficulties to investigators in this field than similar experiments with proteins from other sources. This has probably been due, to a certain extent, to the primary toxicity of many bacterial proteins and, in part, to the difficulty of obtaining a sufficient quantity of concentrated material.

Zinsser and Parker (1), working with a slightly alkaline saline extract of typhoid bacilli, noted its primary toxicity and the quantitative rather than the qualitative differences between passively sensitized and normal guinea pigs when relatively large amounts of antigen were injected; whereas with smaller amounts, they did not obtain acute anaphylactic death. Using the isolated uterus method of Dale (2), they were able to eliminate the error due to toxicity of the antigen, since normal uteri did not react with typhoid antigen. They showed, in this way, that the reactions obtained with bacterial antigens were essentially similar to those obtained with such antigens as horse serum. A critical review of the previous literature on bacterial anaphylaxis is given by these authors.

The experiments reported in this and in the succeeding paper were undertaken primarily to throw some light on the question of the antigenicity of the various fractions isolated from the hemolytic streptococcus. It had already been shown that certainly two and possibly three distinct reactive substances, all non-type-specific, were present in extracts of hemolytic streptococci in addition to the type-specific protein, and that these could be separated chemically and serologically (3). Certain points of interest soon arose with regard to the anaphylactic reactions caused by these fractions, and these observations are recorded. The results presented in this paper were

obtained with the group-reactive nucleoprotein (P) and with the species-specific substance (C) which is probably a carbohydrate; while the results with the type-specific protein (M) and the non-type-specific protein (Y) sometimes associated with it are given in the succeeding paper.

Methods.

The preparation of the extracts used in these experiments has been previously described (3). Briefly, the nucleoprotein (P) was the fraction of NaOH extracts precipitable in the cold with dilute acetic acid, while the probable carbohydrate (C) was obtained from the supernatant fluids after precipitation of the other reactive fractions from their respective extracts. It was freed of protein as far as the small yields permitted. The type-specific (M) protein was obtained from HCl extracts.

The preparation of antibacterial sera was described in detail in a preceding paper and the most satisfactory method of preparing anti-P sera, also described previously (3), is summarized here for convenience. Rabbits were immunized with P by intravenous injections of 1 per cent solutions. Four daily injections of 10 mg. each were made, followed by a 3-day rest, then four more injections of 20 mg. each followed by 3 days of rest as before. A similar third series of injections of 30 mg. doses was given, then a fourth series of 40 mg. doses. If the titer of the test bleeding, taken 7 days later, was unsatisfactory, further series of 40 mg. doses were made until a satisfactory titer was obtained. The animal was then bled and the serum stored in the ice box without preservative.

Guinea pigs were sensitized passively by the intraperitoneal injection of 0.5 cc. of immune serum. Occasionally larger amounts of serum were employed and are thus recorded in the tables. Since it was impossible to obtain enough guinea pigs of exactly the same weight for use in all experiments, animals of approximately the same weight were selected for each experiment. The weights, which usually ranged between 150 gm. and 200 gm., are omitted from the tables. The sensitized animals were tested by intravenous injections of the appropriate extracts at varying intervals after sensitization, as shown in the tables. The smallest amount of extract which regularly caused acute anaphylactic death was called the minimal anaphylactic dose (M. A. D.), following the terminology of Weil (4). Since the concentration of active substances in the extracts was sometimes unknown, the dose could not always be expressed in mg. and was, therefore, in these instances given as M. A. D. for guinea pigs sensitized with the homologous serum. Sometimes both methods of recording the dosage were employed. In those instances in which it was desirable to test the anaphylactic reactions of a sensitized guinea pig with more than one substance, it was found necessary to wait 24 hours between injections in order to give the animal time to recover from the non-specific reduction of reactivity due to the first shock. The necessity for this interval has also been noted by Wells and Osborne in their study of the

relationships of certain plant proteins by means of the anaphylactic reaction (5). Control animals were always injected with doses at least as large as those used for the sensitized guinea pigs, sometimes following an intraperitoneal injection of normal rabbit serum and sometimes without this preliminary injection. They never showed symptoms of shock with the doses employed and survived indefinitely, most of them being kept under observation for several weeks.

A convenient method for making intravenous injections into guinea pigs was brought to our attention by Dr. C. H. Hitchcock. The hair was shaved from the back of the leg and a superficial longitudinal vein was easily laid bare by a small incision in the skin just above the ankle. This vein was small but was satisfactory for intravenous injections with a 25 gauge Yale needle. As many as five separate injections have been made into the same vein, although usually the veins in both legs were used for repeated injections, and occasionally similarly located veins in the forelegs were employed. All intravenous injections reported here were made in this manner.

The results of anaphylactic tests of each reactive fraction injected into guinea pigs sensitized with anti-P and with antibacterial sera are considered in order. The group-reactive P antigen is discussed first since a pure anti-P serum was available for its study. Since Zinsser and Parker reported optimal sensitization after an interval of 5 to 8 days rather than earlier, the following experiment was performed to determine the optimal time for the shocking injection after passive sensitization with this serum.

Experiment 1.—A series of guinea pigs was sensitized with anti-P serum.[1] The M. A. D. of the homologous P was determined at 24-hour intervals throughout a 4-day period. The results are tabulated in Table I.

The experiment showed that the optimal time for passive sensitization with this anti-P serum was not reached until the 2nd or 3rd day, as judged by the size of the M.A.D., and that guinea pigs became less sensitive after the 3rd day.

The next experiment was devised to show the relationships among nucleoproteins from different types of hemolytic streptococci as well as from *Streptococcus viridans* and pneumococcus.

Experiment 2.—Another series of guinea pigs was sensitized with anti-P serum. The M. A. D. of nucleoprotein from strains representing three different types of

[1] In each instance the details of preparation of immune serum are given in the tables; hence they will not be discussed in detail in the text.

hemolytic streptococcus was determined on the day after sensitization by the intravenous injection of varying amounts of a 1 per cent solution. Sensitized

TABLE I.

Anti-P Reactions.

Time Required for Passive Sensitization with an Anti-P Serum.

Guinea pigs sensitized with anti-P serum, R500, from a rabbit immunized with P from hemolytic streptococcus, Strain S39, *Type* S23.

Shocked by intravenous injections of P from homologous strain, S39			
Guinea pig	Days after sensitization	Dose	Result
		mg.	
A-1	1	5.0	+++++
A-2	1	7.5	†5 min.
A-3	2	0.5	+
A-4	2	1.0	†4 min.
A-5	3	0.5	±
A-6	3	1.0	++
A-7	3	2.0	†5.5 min.
A-8	4	2.0	+
A-9	4	5.0	†3.5 min.

20 mg. of P did not shock unsensitized control guinea pigs.

In all tables the following symbols are employed:

− indicates no shock.
±? " trace of shock.
± " slight shock.
+ " mild shock.
++ " moderate shock.
+++ " moderately severe shock.
++++ " severe shock.
+++++ " very severe shock.
† " animal died.

hom. = homologous.
het. = heterologous.
M. A. D. = minimal anaphylactic dose.

guinea pigs were also tested with P from a strain of green streptococcus and with P from pneumococcus, Type III.[2] Animals surviving the first injection were

[2] This protein was an oxidized extract kindly supplied by Dr. Julianelle.

reinjected on the following day with a dose of hemolytic streptococcus P several times the M. A. D. in order to determine whether they had become desensitized. Control animals were not shocked by 20 mg. of P.

TABLE II.

Anti-P Reactions.

Minimal Anaphylactic Dose of Nucleoprotein, P, from Different Strains.

Guinea pigs passively sensitized with anti-P serum, R500, from a rabbit immunized with P from hemolytic streptococcus, Strain S39, *Type* S23.

Guinea pig	Shocked by intravenous injections					
	First test 1 day after sensitization			Second test 2 days after sensitization		
	Antigen: P from strain of *S. hæmolyticus*	Dose	Result	Antigen: P from strain of *S. hæmolyticus*	Dose	Result
		mg.			*mg.*	
B-1	S39 (hom.)	5.0	++	S39 (hom.)	10*	±
B-2	" "	7.5	†3 min.			
B-3	S43 (het.)	5.0	+	" "	10	±?
B-4	" "	7.5	†4.5 min.			
B-5	S3 "	5.0	++**			
B-6	" "	7.5	†4.5 min.			
	P from strains of related species					
B-7	V92 (*S. viridans*)	10.0	+	" "	10	†4 min.
B-8	" " "	15.0	++	S3 (het.)	20	+++
B-9	" " "	20.0	+++	S39 (hom.)	10	++++
B-10	Pneumococcus, Type III	2cc.***	†4 hrs.			

* 10 M. A. D. for 48 hours. See Table I.

** Found dead next day. Small Gram-negative bacillus in heart's blood culture.

*** Concentration of P unknown. Test made 48 hours after sensitization. A control animal was unaffected by the same dose.

Table II shows the results of this experiment. Although the three hemolytic streptococcus proteins represented three distinct serological types, the M.A.D. for guinea pigs sensitized with Serum R500 was the same; while shock, but not death, was produced by considerably

larger doses of green streptococcus protein. A pneumococcus protein injected 48 hours after sensitization resulted in delayed death. Animals surviving shock from the hemolytic streptococcus proteins were completely protected against shock on the following day with 10 mg. of homologous P, although with this interval after sensitization 1 mg. was the M.A.D.; and those surviving shock from the green streptococcus protein were partly protected against the homologous P.

The nucleoproteins were also tested in guinea pigs sensitized with antibacterial sera. Since these sera contained antibodies for all the reactive substances but in different amounts depending on the method of immunization or the individual response of the rabbit, the tests with them were partly incidental to other experiments and were collected as Experiment 3.

Experiment 3.—Guinea pigs sensitized with 0.5 cc. to 1.0 cc. of antibacterial sera were tested 1 to 6 days later by intravenous injections of nucleoprotein P in doses ranging from 4 mg. to 10 mg. The M. A. D. was usually determined. The results are recorded in Table III.

The table is self-explanatory and shows that guinea pigs passively sensitized with antibacterial sera died in acute anaphylactic shock when injected intravenously with 7.5 mg. to 10 mg. of homologous or of heterologous hemolytic streptococcus nucleoprotein. Proteins from the different strains, except possibly from the homologous strain, were approximately equally effective in producing shock. Since homologous P solutions always contained some type-specific protein M, a smaller M.A.D. was to be expected. Precipitin tests, not tabulated here, also showed the presence of P antibodies in all these sera.

Active sensitization was obtained with nucleoprotein antigens in a few instances. After numerous doubtful results, unsuccessful apparently on account of dosage or timing between injections, the following satisfactory experiment in active anaphylaxis was performed.

Experiment 4.—Three guinea pigs were given intravenous injections of P from hemolytic or from green streptococci, as shown in Table IV. 22 days later each guinea pig was reinjected with 20 mg. of hemolytic streptococcus P. Table IV summarizes the experiment.

All three guinea pigs suffered typical anaphylactic shock on reinjection with hemolytic streptococcus proteins. Although the intensity

of the shock varied with different sensitizing antigens and was most marked with the most distantly related P, still the postmortem findings

TABLE III.

Anti-P Reactions.

Effect of Nucleoprotein, P, on Guinea Pigs Sensitized with Antibacterial Sera.

Guinea pig	Sensitized with serum*		Shocked by intravenous injections			
	No.	Cc.	Days after sensitization	Antigen: P from *S. hæmolyticus* strain	Dose	Result
					mg.	
C-1	R323	1.0	6	S39 (het.)	7.5	+
C-2	"	1.0	6	" "	10.0	++++
C-3	"	1.0	6	S60 (hom.)	10.0	†4 min.
C-4	R321	0.5	3	S3 (het.)	10.0	†4 "
C-5**	R261	0.5	1	" "	4.0	−
K-1	"	0.5	1	" "	10.0	†11 min.
C-6	"	1.0	6	S60 "	5.0	++
C-7	"	1.0	6	" "	7.5	†3.5 min.
C-8	"	1.0	6	" "	7.5	†45 "
C-9	"	1.0	6	" "	10.0	†3.5 "
M-2	Q308	1.0	3	S3 "	10.0	†3.5 "
M-3	"	1.0	2	" "	10.0	†50 "
M-6	"	1.0	3	" "	10.0	++
C-10	R446	0.5	1	S43 "	5.0	++
C-11	"	0.5	1	" "	7.5	+++
C-12	"	0.5	1	" "	10.0	†3.5 min.

* Serum R323 was against Strain S43, *Type* S60.
" R321 " " " " " "
" R261 " " " S39, " S23.
" Q308 " " " S23, " "
" R446 " " " S3, " S3.

** Three other guinea pigs in this group received the same dose with the same result.

were typical of anaphylactic death in all instances. This experiment offered sufficient evidence that nucleoprotein antigens from streptococci could sensitize guinea pigs actively.

These experiments confirm the conclusions previously reached as a result of precipitin tests and absorption experiments that the nucleoprotein fraction P is common to hemolytic streptococci and that it is related to similar protein fractions of green streptococci and of pneumococci.

The anaphylactic reactions of the second non-type-specific substance were next investigated. This is referred to as the C substance and is probably a complex carbohydrate chemically similar to the type-specific soluble substances of certain other bacteria. It will be recalled however, that this fraction, while it is species-specific for hemolytic

TABLE IV.

Anti-P Reactions.

Active Sensitization with Nucleoprotein Antigens.

Guinea pig	Sensitizing injection*			Shocking injection* 22 days later			
	Antigen P from strain**	Dose	Result	Weight	Antigen P from strain	Dose	Result
		mg.		*gm.*		*mg.*	
D-1	S43 (*S. hæmolyticus*)	20	−	135	S43 (*S. hæmolyticus*)	20	++++ (†overnight)***
D-2	S3 " "	20	±	186	" " "	20	†50 min.
D-3	V92 (" *viridans*)	20	−	173	" " "	20	†4 "

* Intravenous.

** Strain S43 represents *Type* S60; Strain S3 represents *Type* S3.

*** Lungs distended as in acute anaphylactic death.

streptococci, shows no type specificity whatever in its precipitin reactions. Thus, with antibacterial serum from Rabbit R446 typical disc precipitates were formed in high dilutions of C preparations from all strains of hemolytic streptococcus tested. Consequently, it was important to find out whether this antigen-antibody system could cause anaphylaxis, and the following experiment was performed to determine this point.

Experiment 5.—A series of guinea pigs was sensitized with Serum R446. On the following day the animals were tested with intravenous injections of varying amounts of "purified" C from two heterologous strains of hemolytic streptococcus. The results are given in Table V.

In this experiment typical anaphylactic shock was produced with a substance which is probably a carbohydrate. Titration of C from Strain S43 showed that 0.04 mg. was the M.A.D. for guinea pigs sensitized on the preceding day with Serum R446. Similarly, the M.A.D. of C from Strain S23 was 0.03 mg. The results exactly parallel the precipitin tests recorded in Table I of the preceding paper (3). A

TABLE V.

Anti-C Reactions.

Effect of Two Relatively Pure C Substances on Guinea Pigs Sensitized with Antibacterial Serum with a High Titer of C Precipitins. Determination of Minimal Anaphylactic Dose.

Guinea pigs sensitized with serum from a rabbit immunized with Strain S3, *Type* S3.

Guinea pig	Shocked by intravenous injections 1 day after sensitization		
	"Purified" C from strain	Dose	Result
		mg.	
E-1	S43 (het.)	0.02	++++
E-2	" "	0.04	†3.5 min.
E-3	" "	0.2	†3.5 "
E-4	" "	2.0	†4 "
E-5	S23 "	0.01	±
E-6	" "	0.03	†4 min.
E-7	" "	0.1	†5 "

The three strains used in this experiment represent three distinct types of *S. hæmolyticus:*

S3 represents *Type* S3.
S43 " " S60.
S23 " " S23.

crude C extracted with antiformin from another strain of *Type* S23 also produced typical anaphylactic death in guinea pigs sensitized with this serum. Precipitin tests with this extract were negative for M and for P substances, and paralleled the anaphylaxis results in being positive for the C substance. The results with this extract are not included in the table.

Since the C substance produced undoubted anaphylactic shock, the question of its chemical nature became of even greater interest and

theoretical importance than before. As already pointed out, it was supposed that this fraction was probably a carbohydrate, and the facts supporting this supposition have been considered previously (3), as well as the fact that the chemical analyses do not exclude the possibility that the active material is combined with protein or is protein itself. Exposure of C to active trypsin, however, did not alter its activity in the precipitin test, and the effect of such digestion on the anaphylactic reaction is shown in the following experiment.

TABLE VI.

Anti-C Reactions.

Exposure of C Substance to Trypsin: Effect on Anaphylactic Shock.

Guinea pigs sensitized with Serum R446 from a rabbit immunized with Strain S3, *Type* S3.

Guinea pig	Shocked by intravenous injections 1 day after sensitization		
	Purified C from Strain S23, *Type* S23, treated with:	Dose*	Result
		mg.	
F-1	Inactive trypsin	0.05	+++
F-2	" "	0.075	†3 min.
F-3	Active "	0.05	+++
F-4	" "	0.075	†3.5 min.

* These figures may be somewhat inaccurate on account of the small volumes with which the experiment was performed.

Experiment 6.—"Purified" C substance was subjected to the action of 0.5 per cent trypsin for 50 minutes. The technique of the experiment was the same as that employed in similar experiments described in previous papers (3). The trypsin was shown to be active by a simultaneous experiment in which the type-specific protein M was completely digested under the same conditions. (See Experiment 3 in the following paper.) Titrations of the C substance exposed to inactivated trypsin (the control) and of that exposed to active trypsin were made in guinea pigs sensitized with Serum R446.

Table VI shows that trypsin did not digest the C substance or change its titer in the anaphylactic reaction under conditions which completely destroyed other reactive fractions of the hemolytic streptococcus.

A few additional experiments were made to test the reactivity of C in guinea pigs sensitized with an anti-P serum and with antibacterial sera which showed only a low titer of C antibodies in the precipitin test. Experiment 7 gives some of these results.

Experiment 7.—Two guinea pigs, sensitized 2 days earlier with an anti-P serum, were given intravenous injections of C in doses which represented 3.3 and 20 M. A. D. respectively for animals sensitized with serum potent in C antibody.

TABLE VII.

Anti-C Reactions.
Effect of C Substances on Guinea Pigs Sensitized with:
(1) Anti-P Sera with No C Precipitins.
(2) Antibacterial Sera with Low Titer of C Precipitins.

Guinea pig	Sensitized with serum	Kind of serum*	Shocked by intravenous injections				
			Days after sensitization	Purified C extract from strain	Dose		Result
					Mg.	M.A.D.**	
G-1	R500	Anti-P	2	S23 (hom.)	0.1	3.3	—
G-2	"	"	2	" "	0.6	20.0	—***
G-3	R322	Antibacterial	1	S43 "	0.2	5.0	—***
G-4	R264	"	1	" (het.)	0.2	5.0	—
G-5	"	"	1	S23 (hom.)	0.1	3.3	—***

* R500 was a rabbit immunized with P from Strain S39, *Type* S23.
R322 was a rabbit immunized with Strain S43, *Type* S60.
R264 " " " " " " S39, " S23.

** M. A. D. in terms of Serum R446. See Table V.

*** Intravenous injections of suitable homologous P or M extracts on the following day produced typical anaphylactic death in these animals.

(See Experiment 5.) Since they were not shocked, the sensitiveness of one of these animals, G-2, was tested on the following day by an intravenous injection of nucleoprotein P.

Guinea pigs sensitized with antibacterial sera which had a low titer of C precipitins were also tested with C substances but showed no symptoms of shock, whether the C was obtained from homologous or from heterologous strains. Two of these animals, G-3 and G-5, subsequently received homologous M extracts to prove that they had been really sensitized.

This experiment emphasized the fact that this non-type-specific substance was not the same as the non-type-specific nucleoprotein P since it did not shock Guinea Pigs G-1 and G-2, sensitized with a potent anti-P serum. It also showed that C would shock guinea pigs sensitized with antibacterial sera, only if the serum had a relatively high content of C precipitins. Thus, G-3, G-4, and G-5 showed no shock when given 3 to 5 M.A.D. of C, although subsequent injections of homologous type-specific M extracts proved that this group of animals had been properly sensitized. These results parallel the precipitin reactions. This experiment will be referred to in another connection to show that the C substance is different from another non-type-specific substance (designated Y) which gave cross-reactions with certain antibacterial sera (notably, with Serum R264).

No attempts to sensitize actively with C could be made on account of the small amounts of material available.

DISCUSSION.

The anaphylactic reactions of two of the cell derivatives of *Streptococcus hæmolyticus* paralleled in every instance the precipitin reactions previously reported (3). The similarity of nucleoproteins (P) from different types as well as from different strains of hemolytic streptococci was again evident; while the partial relationships of P from related species of bacteria, such as *Streptococcus viridans* and pneumococcus, were also apparent. These conclusions are based on passive anaphylactic experiments with anti-P and with antibacterial sera and on active sensitization with P which was also accomplished.

The species-specific C substance was also effective in producing anaphylactic death in passively sensitized guinea pigs, but only if the serum showed a high titer of the non-type-specific C precipitins. Amounts as small as 0.03 mg. to 0.04 mg. were sufficient to cause death in such animals. This is an instance in which a substance, probably carbohydrate in nature, causes passive anaphylactic shock, an interesting finding in view of its chemical nature and the failure to immunize with it. It seems to be a hapten capable of reacting with antibody but not capable of stimulating antibody formation. Insufficient quantities have been available to determine its capacity to produce active anaphylaxis. A similar result has been reported since the conclusion of these experiments by Tomcsik (6) who obtained from *B. lactis*

aerogenes 5 gm. of a specific substance which was largely carbohydrate, although he could not free it of 0.9 per cent nitrogen. 0.03 mg. of this product was the minimal anaphylactic dose for guinea pigs passively sensitized with antibacterial serum, a figure strikingly similar to that obtained with the probable carbohydrate of the hemolytic streptococcus. Tomcsik's material was non-antigenic in that it did not produce active anaphylaxis in guinea pigs nor antibodies in rabbits into which it was injected. His work is analogous to that reported here with the C substance; and in both instances the final proof that the material is pure carbohydrate is still lacking, although the amount of nitrogen present in one minimal anaphylactic dose is certainly small. In the case of the streptococcus C, which contained approximately 4 per cent nitrogen, the minimal anaphylactic dose, therefore, contained only about 0.000001 gm. of nitrogen. In the present anaphylactic experiments, additional evidence that C is not a protein is the failure to digest it with trypsin; and its failure to shock guinea pigs passively sensitized with anti-P serum is further evidence that it is distinct from the non-type-specific nucleoprotein, P.

SUMMARY.

The anaphylactic reactions of two non-type-specific fractions of hemolytic streptococcus extracts parallel the precipitin reactions. The nucleoprotein, P, is a true antigen, in that it stimulates antibody production in rabbits, as shown before, and produces anaphylactic shock in guinea pigs actively as well as passively. The probable carbohydrate, C, on the other hand, does not induce antibody formation in rabbits, so far as known at present, but does produce typical anaphylactic shock in guinea pigs passively sensitized with antibacterial serum provided the serum shows a high titer of C precipitins. This is an instance of a hapten, probably carbohydrate in nature, causing anaphylactic shock in passively sensitized guinea pigs.

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THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

V. Anaphylaxis with the Type-Specific Substance.

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The anaphylactic reactions caused by the group-reactive nucleoprotein P, and by the species-specific substance, C, obtained from extracts of hemolytic streptococcus have been shown in the preceding paper to parallel their precipitin reactions (1). Thus equivalent amounts of nucleoproteins derived from different types of hemolytic streptococci caused acute anaphylactic death in guinea pigs passively sensitized with anti-P serum prepared with nucleoprotein from any of these types. Moreover, nucleoproteins from related species of bacteria, *Streptococcus viridans* and pneumococcus, also caused anaphylactic death if injected into similarly sensitized guinea pigs in sufficiently large doses. This partial relationship had already been observed in precipitation and absorption experiments (2). The nucleoproteins were also capable of sensitizing guinea pigs in active anaphylactic experiments. The other non-type-specific fraction, the species-specific C substance, which seemed to be non-protein and probably carbohydrate, was also effective in producing acute anaphylactic death in passively sensitized guinea pigs. Only those antibacterial sera which showed a high titer of C antibodies in the precipitin test, however, were under the conditions of the experiments capable of sensitizing guinea pigs passively to shock with the C substance. As in the precipitin reaction, so also in the anaphylactic reaction, the C substance showed no type specificity but gave cross-reactions with sera prepared against all types of hemolytic streptococci. While the chemical data were not sufficient to prove conclusively that this substance was a carbohydrate, still they indicated that this was the case. They were substantiated by the failure of trypsin or of pepsin to affect

either the precipitin titer or the minimal anaphylactic dose of C. These two non-type-specific substances extracted from hemolytic streptococci differed both chemically and in their failure to cause reciprocal precipitin or anaphylactic reactions with their respective antisera.

The present experiments are concerned with the anaphylactic reactions of the type-specific protein, M, and with certain cross-reactions observed with some of the extracts containing M which may be due to a third non-type-specific fraction, Y.

Methods.

The preparation of extracts and of antisera has been described, and the technique of the anaphylactic test is the same as that employed with the non-type-specific fractions (1).

The protein fraction, M, which gave type-specific precipitin reactions, was tested for type-specific anaphylactic reactions as shown in Experiment 1.

Experiment 1.—Guinea pigs, sensitized with antibacterial serum, were tested with intravenous injections of homologous and of heterologous HCl extracts which contained the type-specific M. Surviving guinea pigs were reinjected on the following day with homologous M. The M.A.D. for each extract was determined, and doses were recorded as approximate multiples of the M.A.D. with a definite homologous serum. Control unsensitized guinea pigs were never shocked by these extracts. Table I shows some of the type-specific reactions.

Guinea pigs sensitized with eight different antibacterial sera showed only slight reactions when tested with intravenous injections of 2 to 50 M.A.D. of heterologous M, although the controls, similarly sensitized, died in acute anaphylactic shock when given 1 or 2 M.A.D. of homologous M. The animals surviving heterologous M, either succumbed to injection of homologous M on the following day or suffered a severe but sublethal shock. The increase in amount of homologous M required to produce death following shock with heterologous M was probably due to the presence in these extracts of traces of non-type-specific substances as impurities. Such an experiment was evidence of type-specific anaphylactic shock with the M fraction. A micro Kjeldahl nitrogen determination made on the most active lot

of HCl extract from Strain S43 showed that the M.A.D. for guinea pigs passively sensitized with homologous Serum R322 was 0.2 mg. of protein.

Active sensitization with HCl extracts was attempted; but, although doses and intervals between injections were varied considerably, no positive results were obtained. In view of the difficulty of finding the right conditions for sensitization with P, however, these negative results were not considered conclusive.

Occasional sera were encountered which gave non-type-specific anaphylactic, as well as precipitin, reactions with relatively purified HCl extracts from Strain S43. Since it was obvious that the type-specific M was not completely isolated but that traces of the other reactive fractions were almost certainly present, it was possible that these results were due to reactions of the latter with their respective antibodies which are usually present in antibacterial sera. Experiment 2 was performed to test this hypothesis.

Experiment 2.—Five guinea pigs, I-1 to I-5, sensitized with Type S23 antibacterial sera received intravenous injections of HCl extract from a strain of heterologous type, as shown in Table II. All these animals died in typical anaphylactic shock. In order to determine whether this cross-reaction was due to traces of the common nucleoprotein P, Guinea Pigs I-6 to I-12 were sensitized with a highly potent anti-P serum and were given intravenous injections of the extract used in the first part of the experiment. Such slight symptoms resulted that P was eliminated as the cause of the cross-reactions in I-1 to I-5. The extract was next tested for the presence of the other known commonly reactive substance, C, by injecting it into guinea pigs sensitized with an antibacterial serum of high potency for C antibody. Since none of these animals showed more than very slight symptoms, the common C substance was also eliminated as the cause of the cross-reactions.

All surviving guinea pigs (I-6 to I–16) were tested again, usually on the following day, with suitable homologous extracts. Typical anaphylactic death resulted, except in the cases of I-6 and I-7 which suffered + + + and + + shock respectively, thus showing that the animals had been effectively sensitized.

Table II shows the cross-reactions of occasional *Type* S23 antibacterial sera with certain HCl extracts from another type. The possible explanation that traces of the common substances, P and C, were responsible for these non-type-specific reactions was eliminated in each instance by testing the extract for the presence of P and of C

TABLE I.

Anti-M Reactions.

Type-Specific Anaphylactic Shock with Antibacterial Sera and HCl Extracts.

Guinea pig	Sensitized with serum*		Shocked by intravenous injections				
			Test No.	Days after sensitization	HCl extract from strain**	Dose M.A.D.‡	Result
	No.	Cc.					
H-1	R323	1.0	1	6	S43 (hom.)	20.0	†4.5 min.
H-2	"	1.0	1	6	S39 (het.)	2.0	±
			2	7	S43 (hom.)	1.0	†4 min.
H-3	R321	0.5	1	1	S43 (hom.)	1.0	†4.5 min.
H-4	"§	0.5	1	1	S39 (het.)	5.0	++
			2	3	" "	10.0	±
			3	4	S43 (hom.)	2.0	†4 min.
H-5	Q866	0.5	1	2	S43 (hom.)	2.0	†4 min.
H-6	"	0.5	1	2	S39 (het.)	2.5	±
			2	3	S43 (hom.)	2.0	++
H-7	Q864	0.5	1	2	S43 (hom.)	2.0	†3 min.
H-8	"	0.5	1	2	S39 (het.)	2.5	±
			2	3	S43 (hom.)	4.0	++
H-9	R322	0.5	1	2	S43 (hom.)	2.0	†4.5 min.
H-10	"	0.5	1	2	S39 (het.)	2.5	±?
			2	3	S43 (hom.)	6.0	+++
H-11	R324	0.5	1	2	S43 (hom.)	2.0	†4 min.
H-12	"	0.5	1	2	S39 (het.)	2.5	±?
			2	3	S43 (hom.)	6.0	†4 min.
H-13	Q613	1.0	1	1	S39 (hom.)	1.0	†7 min.
H-14	"	1.0	1	1	S43 (het.)	50.0	++
			2	3	S39 (hom.)	2.0	†3.5 min.
H-15	Q317	1.0	1	2	S39 (hom.)	1.0	++++
			2	3	" "	2.5	+++
H-16	"	1.0	1	2	S43 (het.)	2.0	±
			2	3	S39 (hom.)	2.5	+++

by injecting it into guinea pigs sensitized with sera of known high potency for P and for C antibodies. It was shown previously in the reciprocal experiment[1] that "purified" C did not shock guinea pigs sensitized with one of the sera which gave cross-reactions in the present experiment (Serum R264 used for I-5). This is further evidence that C is not the cause of the cross-reactions in the present instance. Since this hypothesis proved, therefore, untenable, it was necessary to assume either (1) that still another non-type-specific substance was present in the antigenic complex of the hemolytic streptococcus, or else (2) that the M substance was not strictly type-specific but that it might be nearly enough related to M from a different type to cross occasionally with an antibody which is *chiefly* specific for the latter. Considerable evidence has been accumulated indicating that the second hypothesis is incorrect. A comparison of the M.A.D. of different lots of extracts in terms of animals sensitized with homologous type serum and of others sensitized with heterologous type

[1] See Paper IV of this series, Table VII, G-4 and G-5.

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* Sera R321, R322, R323, and R324 were against Strain S43, *Type* S60.
" Q864 and Q866 " " " S60, " "
" Q613 " Q317 " " " S23, " S23.

** Strain S43 represents *Type* S60.
" S39 " " S23.

‡ M.A.D. of S43 extract in terms of Serum R321.
" " S39 " " " " " R261 or R264.

§ 6 other guinea pigs sensitized with this serum and tested with varying combinations of these 2 antigens reacted in the same way.

In all tables the following symbols are employed:

−	"	no shock.
±?	"	trace of shock.
±	"	slight shock.
+	"	mild shock.
++	"	moderate shock.
+++	"	moderately severe shock.
++++	"	severe shock.
+++++	"	very severe shock.
†	"	animal died.

hom. = homologous.
het. = heterologous.
M.A.D. = minimal anaphylactic dose.

TABLE II.

Anti-Y Reactions.

Non-type-Specific Anaphylactic Shock with Certain Antibacterial Sera and HCl Extracts.

Guinea pig	Sensitized with serum No.*	Shocked by intravenous injections of HCl extract from Strain S43, *Type* S60			
		Days after sensitization	M.A.D. in terms of serum against homologous *Type* S60	M.A.D. in terms of serum against heterologous *Type* S23	Result
Antibacterial sera giving cross-reactions with HCl extracts from a strain of another type					
I-1	Q308	2	2	2	†4 min.
I-2	"	4	20	1.3	†4 "
I-3	R261	6	2	2	†4.5 "
I-4	"	1	2	2	†3 "
I-5	R264	2	2	2	†6 "
Pure Anti-P sera					
I-6	R500	6	2.5	2.5	+
I-7	"	3	10	10	±?
I-8	"	1	10	10	+
I-9	"	1	10	10	+
I-10	"	1	2	2	+
I-11	R594	1	2	2	±?
I-12	"	3	10	10	−
Antibacterial serum containing C antibody					
I-13	R446	1	1.3	1	+
I-14	"	2	15	1	+?
I-15	"	1	1	1−	±
I-16	"	2	?**	3	±

* Guinea Pigs I-1, -2, -3, -5, -6, -11, -12 were sensitized with 1 cc. of serum; all others with 0.5 cc.

R500 was an anti-P serum against P from Strain S39, *Type* S23.

R594 " " " " " " " " S43, " S60.

All others were antibacterial sera:

R446 was against Strain S3, *Type* S3

Q308 " " " S23, " S23.

R261 " " " S39, " "

R264 " " " " " "

** HCl extract from Strain S39, *Type* S23, given to this animal.

sera shows wide variation. For example, with the extract used to test Guinea Pig I-1, the M.A.D. in terms of *Type* S23 and *Type* S60 sera was the same, while with an extract from the same strain prepared at another time (used for I-2) the M.A.D. in terms of heterologous *Type* S23 serum was fifteen times the M.A.D. in terms of homologous *Type* S60 serum. This difference in degree of heterologous activity of different lots of extract seems significant in indicating that M itself is not responsible for the cross-reaction, but rather that some non-type-specific substance, also extracted at times by HCl, is the cause of these atypical reactions.

The following experiment shows the effect of tryptic digestion on the anaphylactic reactions of this so called Y substance as well as its effect on the type-specific M.

Experiment 3.—A concentrated HCl extract from Strain S43 was digested with 0.5 per cent trypsin for 50 minutes with the technique previously described.[2] Controls with heated trypsin were included. Guinea Pig O-1, sensitized with serum prepared against Strain S43, served as control and died in acute anaphylactic shock on injection of 1 M.A.D. of the undigested extract (containing heated trypsin). Guinea Pig O-2, on the contrary, sensitized in the same way, was unaffected by the same dose of digested extract, although it was proved sensitive by injection on the next day of the same amount of untreated extract which resulted in typical anaphylactic death.

The same solution, known to give cross-reactions characteristic of the non-type-specific Y, was tested in Guinea Pigs O-3 and O-4, sensitized with serum prepared against a heterologous type strain. The control, O-3, died of anaphylactic shock when given 1 M.A.D. of the undigested extract, while O-4 suffered no shock from the same amount of digested extract. On the following day, however, O-4 was killed by 1 M.A.D. of untreated extract, thus proving that it was sensitive. Table III shows these results.

The results of this experiment were clear. Tryptic digestion destroyed the type-specific M contained in S43 HCl extract, as shown by the failure of the digested material to shock O-2, which had been sensitized with the homologous serum. The animal was sensitive, for it was killed by subsequent injection with untreated extract. The control animal, O-1, which received the undigested (heated trypsin) extract, died in 3 minutes. Similar results were obtained when the

[2] Experiment 3 described in Paper II of this series.

digested and the undigested S43 HCl extracts were tested in guinea pigs sensitized with heterologous Serum R264: the digested extract did not affect O-4 (although subsequent injection with untreated extract caused acute anaphylactic death), while the undigested control extract was still effective in shocking Guinea Pig O-3. Like all

TABLE III.

Tryptic Digestion of HCl Extracts Containing M and Y; Effect on Anaphylactic Shock.

Guinea pig	Sensitized with serum No.*	Shocked by intravenous injections of 1 to 1.5 M.A.D.					
		Test No.	Days after sensitization	HCl extract		Result	Probable chief cause of reaction
				from strain:	treated with:		
To show tryptic digestion of M							
O-1	R322	1	1	S43 (hom.)	Inactive trypsin	†3 min.	M
O-2	"	1	1	" "	Active trypsin	—	—
		2	2	" "	—	†4 min.	M
To show tryptic digestion of Y							
O-3	R264	1	1	S43 (het.)	Inactive trypsin	†3.5 min.	Y
O-4	"	1	1	" "	Active trypsin	—	—
		2	2	" "	—	†14 min.	Y

* Guinea pigs sensitized with 0.5 cc. of serum.

Serum R322 was an antibacterial serum from a rabbit immunized with Strain S43, *Type* S60; Serum R264 was an antibacterial serum from a rabbit immunized with Strain S39, *Type* S23

the other anaphylactic tests, this corroborated the previous experiments, the results of which were tested only by the precipitin reaction, and was further evidence that these two fractions were proteins. It did not, however, serve to show that this extract really contained two substances. The following absorption experiment differentiated them in such a way as to remove the doubt as to the existence of Y.

Experiment 4.—Three guinea pigs, sensitized with untreated serum from Rabbit R261, were used as controls on the activity of the extracts: J-1 died when tested with HCl extract from a heterologous strain, while J-2 and J-3 also died when tested with HCl extract from the homologous strain. Three other guinea pigs, J-4 to J-6, were sensitized with the same serum which had been absorbed by the technique previously described (2) with a strain (S24) known to be heterol-

TABLE IV.

Antibacterial Serum, R261, Rendered Type-Specific in Its Anaphylactic Reactions by Absorption with Heterologous Bacteria, Strain S24.

Guinea pig	Sensitized with Serum R261*		Shocked by intravenous injections				
	Cc.	Preliminary treatment of serum	Test No.	Days after sensitization	HCl extract from strain	Dose M.A.D. **	Result
J-1	0.5	None (control)	1	1	S43 (het.)	2	†3 min.
J-2	0.5	" " ‡	1	1	S39 (hom.)	1—?	†60 "
J-3	0.5	" " §	1	1	" "	1	†5 "
J-4	0.9	Absorbed	1	1	S39 (hom.)	1—?	+++
J-5	0.9	"	1	1	S43 (het.)	2	—
			2	2	S39 (hom.)	2—	†3.5 min.
J-6	0.45	"	1	1	S43 (het.)	10	—
			2	2	S39 (hom.)	2+	†5.5 hrs.

* Serum R261 was from a rabbit immunized with Strain S39, *Type* S23.

** All M.A.D. in terms of Serum R261.

‡ J-2 controls J-4 and J-5.

§ J-3 " J-6.

ogous to all the other strains used in this experiment. J-4, tested with the homologous HCl extract, suffered severe shock but recovered since too small a test dose was used; J-5 and J-6 were first tested with the same heterologous extract which had killed J-1, but they were unaffected by it. The next day, however, they succumbed to homologous HCl extract, thus showing that they had been sensitized. All necessary controls were negative. Table IV summarizes the results.

The serum used in this experiment sensitized animals to shock with heterologous HCl extracts, but after it was absorbed with entirely

heterologous bacteria, it induced only type-specific anaphylactic reactions. Guinea Pigs J-5 and J-6, sensitized with absorbed serum, withstood respectively 2 and 10 M.A.D. of heterologous HCl extract. Subsequent injection on the following day with the homologous extract resulted in acute anaphylactic death in the case of J-5 and in delayed death in the case of J-6, which, however, had been sensitized with only half as much absorbed serum as the other animals. The absorption method, therefore, eliminated the confusing cross-reactions and brought out type-specific anaphylactic as well as precipitin reactions, thus showing that M and V must be different substances.

Tables V to VIII, inclusive, show how much was accomplished towards demonstrating type-specific anaphylactic reactions in this serum and in others which crossed with heterologous HCl extracts, by the method of desensitizing guinea pigs with extracts of various kinds and subsequently testing with the homologous extract containing M. This *in vivo* saturation of antibodies was comparable, in part, to the *in vitro* absorption described above. The results are somewhat complicated in this instance, however, by the necessity of using extracts which, although they contained one reactive substance in excess, contained in addition traces of others as impurities. Table V shows desensitization with one of these extracts.

Experiment 5.—Guinea Pigs K-1, K-2, and K-3 were sensitized with antibacterial serum, R261, the same serum used in absorption experiment, No. 4. On the following day at hourly intervals, five subcutaneous injections each of 10 mg. of NaOH extract (chiefly P) from heterologous Strain S3 were given to K-2 and K-3. 1 hour after the last injection, 20 mg. of this heterologous extract, given to K-2 intravenously, caused no symptoms, although 10 mg., given to the sensitized but otherwise untreated control K-1, caused typical anaphylactic death. At the same time, 20 mg. of the homologous NaOH extract (containing M as well as P) caused immediate death when given intravenously to the treated K-3.

This simple experiment showed that a sensitized guinea pig (K-2) was desensitized by subcutaneous injections of P so that it no longer reacted to twice the amount of P required to kill a similarly sensitized animal (K-1) which had not received these subcutaneous injections. Guinea Pig K-3, desensitized in the same way as K-2, was, however, killed by 20 mg. of homologous NaOH extract. Since this always contained the type-specific M, in addition to P, the experiment showed

that desensitization to P was accomplished by subcutaneous injections of P without desensitizing the animal to homologous M.

The effect of such desensitization with NaOH extracts on subsequent shock with HCl extracts was tested in Experiment 6.

TABLE V.

Antibacterial Serum, R261, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with Heterologous NaOH Extract on Subsequent Shock with Heterologous and with Homologous NaOH Extract.

Guinea pigs sensitized with 0.5 cc. of serum, R261, from a rabbit immunized with Strain S39, *Type* S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections 1 day after sensitization				
		NaOH extract from strain	Dose	Result	Probable chief cause of reaction	Remarks
			mg.			
K-1	None (control)	S3 (het.)*	10**	†11 min.	P	
K-2	5 subcutaneous injections totaling 50 mg. (5 M.A.D.) of NaOH extract from heterologous strain, S3, 1 day after sensitization	" "	20	±?‡	—	Desensitized to P
K-3	Same as K-2	S39 (hom.)	20	†4 min.	M	Not desensitized to hom. M

* Strain S3 represents *Type* S3.

** 1 M.A.D.

‡ A subsequent test on this animal with homologous M (HCl extract), following a test immaterial to this experiment, resulted in severe shock.

Experiment 6.—Guinea Pigs L-1 to L-4 were sensitized with another Type S23 serum which induced cross-anaphylactic reactions with HCl extracts from a heterologous type. L-1 and L-2 were controls on the activity of the extracts: L-1 succumbed to an intravenous injection of heterologous HCl extract, and L-2 to homologous HCl extract. 2 days after sensitization at hourly intervals L-3 and L-4 were given subcutaneous and intraperitoneal injections of NaOH ex-

tracts from a heterologous strain increasing in dosage from 5 mg. to 16.5 mg. until a total of 50 mg. had been administered. The following day the heterologous HCl

TABLE VI.

Antibacterial Serum, R264, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with NaOH Extract, from a Strain Heterologous to the Serum and to All Test Extracts, on Subsequent Shock with Heterologous and with Homologous HCl Extracts.

Guinea pigs sensitized with 0.5 cc. of serum, R264, from a rabbit immunized with Strain S39, *Type* S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections							
		Test No.	Days after sensitization	HCl extract from strain*	Dose M.A.D. in terms of serum No.** R 264	Dose M.A.D. in terms of serum No.** R 321	Result	Probable chief cause of reaction	Remarks
L-1	None (control)	1	2	S43 (het.)	2	2	†6 min.	Y	
L-2	" "	1	1	S39 (hom.)	1	0	†5 "	M	
L-3	5 subcutaneous and intraperitoneal injections, totaling 50 mg. (5 M.A.D.) of NaOH extract from heterologous Strain S3, 2 days after sensitization	1	3	S43 (het.)	2	2	—	—‡	L-3 and L-4 show a certain amount of desensitization to Y but not to hom. M
L-4	Same as L-3	1	3	" "	4	4	+++	Y	
		2	4	S39 (hom.)	5	0	†3.5 min.	M	

* Strain S3 represents *Type* S3.
" S43 " " S60.
" S39 " " S23.

** Serum R264 was against Strain S39, *Type* S23.
" R321 " " " S43, " S60.

‡ Found dead next morning; blood culture sterile.

extract was titrated by giving the desensitized guinea pigs injections of 2 and 4 M.A.D. of this extract. The smaller dose produced no shock in L-3, and the larger dose produced moderately severe, but not fatal, shock in L-4. The latter

animal was killed by 5 M.A.D. of homologous HCl extract given intravenously the next day.

This experiment showed that NaOH extracts contained substances which could desensitize guinea pigs to a certain extent to shock with heterologous HCl extract containing the non-type-specific Y. It seems probable that this is due to the presence of the Y substance in both extracts. A similar experiment was performed with another *Type* S23 serum, as shown in Table VII.

Experiment 7.—This experiment differed from Experiment 6 in the use of *Type* S23 serum prepared against another strain. The first four animals sensitized with this serum served as controls on the activity of the extracts. M-5 was desensitized by subcutaneous and intraperitoneal injections of NaOH extract from an entirely heterologous strain by the same method as L-3 and L-4 in the last experiment. The following day it survived an injection of more than 2 M.A.D. of heterologous HCl extract but was killed on the next day by the homologous HCl extract. M-6 was desensitized by one intravenous injection of 10 mg. of heterologous NaOH extract, a usually fatal dose, which did not kill this animal. The following day it showed very slight symptoms on injection of heterologous HCl extract but died the next day after an injection of homologous HCl extract. See Table VII for details of the experiment.

In this experiment, which is similar to Experiment 6, desensitization with entirely heterologous NaOH extract given subcutaneously and intraperitoneally or intravenously, resulted in some degree of desensitization with respect to the heterologous HCl extract which previously had killed animals sensitized with this serum. These animals, however, were not desensitized to the homologous HCl extract, for both were killed by intravenous injections of homologous HCl extracts, which in the case of M-6 was in a dose comparable in size to the dose of heterologous HCl extract used on the preceding day. Here again the desensitization was probably due to Y contained in both NaOH and HCl extracts.

In the next experiment the animals were desensitized by preliminary injections of heterologous HCl extract instead of NaOH extract and the effect of desensitization tested for both these extracts as well as for the homologous HCl extract.

Experiment 8.—A series of guinea pigs was sensitized with 1 cc. of the same serum used in the last experiment. Two of these animals served as controls on

TABLE VII.

Antibacterial Serum, Q308, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with NaOH Extract, from a Strain Heterologous to the Serum and to All Test Extracts, on Subsequent Shock with Heterologous and with Homologous HCl Extracts.

Guinea pigs sensitized with 1 cc. of serum, Q308, from a rabbit immunized with Strain S23, *Type* S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections							
		Test No.	Days after sensitization	Extract from strain*	Kind of extract	Dose M.A.D. in terms of serum No.** Q308	Dose M.A.D. in terms of serum No.** R322	Result	Probable chief cause of reaction
M-1	None (control)	1	2	S43 (het.)	HCl	1	7.5	†4 min.	Y
M-2	" "	1	3	S3 "	NaOH	1±‡	1	†3.5 "	P
M-3	" "	1	2	" "	"	1±	1	†50 "	"
M-4	" "	1	1	S39 (hom.)	HCl	1	0	†4 "	M
M-5	5 subcutaneous and intraperitoneal injections, totaling 150 mg. (15 M.A.D.), of NaOH extract from heterologous Strain S3, 1 day after sensitization	1	2	S43 (het.)	"	2.7	20	—	—
		2	3	S39 (hom.)	"	10	0	†3.5 min.	M
M-6	None (desensitized by intravenous injections)	1	3	S3 (het.)	NaOH	1±‡	1	++	P
		2	4	S43 "	HCl	1.3	10	±	Y
		3	5	S39 (hom.)	"	1.5	0	†30 min.	M

* Strain S3 represents *Type* S3.
" S43 " " S60.
" S39 " " S23.

** Serum Q308 was against Strain S23, *Type* S23.
" R322 " " " S43, " S60.

‡ 10 mg.

the effectiveness of the antigens; N-1 was killed by an intravenous injection of 2 M.A.D. of heterologous S43 HCl extract, and M-4 by 1 M.A.D. of homologous S39 HCl extract.

Guinea Pig N-3 was desensitized 2 days after sensitization by subcutaneous injections of 15 M.A.D. (in terms of both homologous and of heterologous sera) of heterologous S43 HCl extract. The next day this animal gave practically no reaction to an intravenous injection of 4 M.A.D. of the same extract, while it

TABLE VIII.

Antibacterial Serum, Q308, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with Heterologous HCl Extract on Subsequent Shock with Heterologous NaOH Extract and with Heterologous and Homologous HCl Extracts.

Guinea pigs sensitized with 1 cc. of serum, Q308, from a rabbit immunized with Strain S23, *Type* S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections: Test No.	Days after sensitization	Extract from strain*	Kind of extract	Dose* M.A.D. in terms of serum No. Q308	Dose* M.A.D. in terms of serum No. R322	Result	Probable chief cause of reaction
N-1	None (control)	1	2	S43 (het.)	HCl	2	2	†4 min.	Y
M-4	" "	1	1	S39 (hom.)	"	1	0	†4 "	M
N-3	3 subcutaneous injections, totaling 15 M.A.D. of S43 HCl extract 2 days after sensitization	1	3	S43 (het.)	"	4	4	±	Y
		2	4	S39 (hom.)	"	5	0	†4 min.	M
N-4	None (desensitized by intravenous injections)	1	2	S43 (het.)	"	0.7	5	++	Y
		2	4	" "	"	4.7	35	−	−
		3	5	S3 "	NaOH	2±	2**	−	−
		4	6	S39 (hom.)	HCl	5	0	†22 min.	M

* See foot-notes * and **, Table VII.

** 20 mg. See Guinea Pigs M-2, M-3, and M-6, Table VII, for controls on the M.A.D. of this extract.

died in 4 minutes on the following day after an intravenous injection of 5 M.A.D. of homologous S39 HCl extract.

Guinea Pig N-4 was desensitized by intravenous injections of heterologous S43 HCl extract. 2 days after sensitization, 0.7 M.A.D. of this solution caused ++ shock; 2 days later 4.7 M.A.D. of the same solution produced no effect; and the next day, 2 M.A.D. of NaOH extract from an entirely different strain, S3, caused

no symptoms. On the following day, however, the animal died a typical anaphylactic death, though delayed to 22 minutes, following an intravenous injection of 5 M.A.D. of homologous S39 HCl extract. Table VIII gives these results.

This experiment was the reciprocal of Experiments 6 and 7. In the latter, heterologous NaOH extract was used to desensitize against HCl extract from a heterologous strain of still another type. In Experiment 8 the reverse procedure was employed: the heterologous HCl extract was used to desensitize against the NaOH extract. In both instances, a certain degree of reciprocal desensitization was obtained without as much desensitization for the homologous HCl extract. These sera, with which it had previously been impossible to demonstrate type-specific anaphylactic reactions, were, therefore, rendered type-specific in that the desensitized animals reacted slightly, or not at all, with several multiples of the M.A.D. of heterologous extracts and were killed by similar, or even smaller doses, of homologous extracts.

If no other evidence were available, the cross-desensitization between NaOH extracts and HCl extracts could be explained as due to the presence in both solutions of the non-type-specific substances, C and P, but it has already been shown in Experiment 2 that the cross-reactions of the HCl extracts cannot be due to C or to P. Since the desensitization does not prevent the reaction with the homologous extracts, it seems improbable that it could be accounted for as non-specific reduction of reactivity such as is occasioned by injection of peptones or other foreign proteins. The correct explanation, therefore, seems to be the assumption of this other non-type-specific substance, Y, in extracts of the hemolytic streptococcus.

DISCUSSION.

Type-specific anaphylactic reactions were easily obtained with the protein, M, (HCl extract) when injected into guinea pigs passively sensitized with most of the antibacterial sera used in these experiments. Four sera produced by immunizing rabbits with strains of one type were, however, encountered, which caused non-type-specific anaphylactic death in passively sensitized guinea pigs when HCl extract from a strain of another type was used for the intoxicating injection. In order to obtain type-specific anaphylactic shock in guinea pigs pas-

sively sensitized with these sera, it was necessary either (1) to absorb the serum with heterologous bacteria or (2) to desensitize the passively sensitized guinea pigs with heterologous extracts. Since the HCl extract responsible for these atypical reactions did not cause reactions in guinea pigs sensitized with potent anti-P sera or with sera potent in C antibody and, conversely, since the C substance did not cause reactions with these atypical antibacterial sera, it was necessary to assume that another non-type-specific substance was present in HCl extracts and in the hemolytic streptococcus itself. The additional fact that this so called Y substance was digested by trypsin, while C was not, eliminated the possibility that C and Y were the same substance. Reciprocal desensitization experiments with heterologous NaOH extracts and with the HCl extract which gave the atypical crossing, showed some cross-desensitization for these heterologous extracts though not for homologous, a fact probably explainable as due to the presence of varying mixtures of all the non-type-specific substances in these extracts.

Active sensitization was not accomplished with M although numerous attempts were made under varying conditions, a result which agrees with another negative result, namely, the failure so far to immunize rabbits with this substance. In both instances, however, the failure must be taken with reserve, since it is possible that some change in technique might reverse the result. Active sensitization with Y (as contained in HCl extracts) was not observed.

A certain amount of additional information as to the nature and relationships of some of the reactive substances contained in the hemolytic streptococcus has been obtained by the use of the anaphylactic reaction. It is certain that the type-specific M produced typical anaphylactic shock in passively sensitized guinea pigs. *A priori*, this fact might be considered an indication that the M substance could function as an antigen in inducing antibody production: actually, all immunization experiments with rabbits and active sensitization experiments with guinea pigs yielded negative results. But in view of the additional evidence that C, which seems to be carbohydrate in nature, also shocked passively sensitized guinea pigs and never gave evidence of antibody stimulation, it seems probably that these two substances are haptens which react with antibodies produced by the

intact bacteria, when tested by means of the precipitin reaction and the passive anaphylactic reaction, but that they have no power to elicit antibodies themselves.

The facility with which acute anaphylactic death was produced by injecting bacterial extracts into passively sensitized guinea pigs was striking. Several factors are probably involved. Many investigators have found it necessary to resort to the Dale method with the excised uterus on account of the primary toxicity of the extracts to be tested. Zinsser and Parker (3) found this true in working with extracts of typhoid bacilli, and later Zinsser and Mallory (4) had the same experience with pneumococcus extracts and even with that method found that the margin between the dose which caused reactions in the normal uterus and that which caused it in the sensitive uterus was not so great as in anaphylaxis with egg albumin or with horse serum. With the hemolytic streptococcus, however, such difficulties fortunately were not encountered, and normal guinea pigs did not react to many multiples of the test doses. The fact that relatively large amounts of concentrated extracts were available and that the serological activity, as well as the number and kind of substances present, was capable of titration in most instances contributed to the success of these experiments. An analysis of the antibodies present in the immune sera used for passive sensitization was also made by means of the precipitin test, with the result that sera with known antibody content were used in the anaphylactic experiments and the control of the results was, therefore, increased.

SUMMARY.

1. Type-specific anaphylactic shock was produced with HCl extracts of *Streptococcus hæmolyticus* in guinea pigs passively sensitized with antibacterial sera.

2. With occasional sera and certain HCl extracts, type-specific shock was not produced unless the serum was first absorbed with heterologous bacteria or unless the guinea pigs were desensitized with heterologous extracts before testing with the homologous extract. The findings indicated that this was due to the presence of the non-type-specific substance which has been provisionally designated as Y.

3. Tryptic digestion destroyed the ability of HCl extracts containing

the type-specific M substance and the non-type-specific Y substance to produce anaphylactic shock in passively sensitized guinea pigs.

4. Active sensitization was not accomplished with the type-specific M. It seems probable, therefore, that this substance is a hapten, reacting with antibodies but not stimulating their production after separation from the bacterial cell.

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THE PROPERTIES OF THE BACTERICIDAL SUBSTANCE IN MILK.

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From the work of many it has been established that fresh raw milk will inhibit for a time the growth of a variety of organisms.

Thus Hesse (1) noted that raw milk suppressed the growth of *B. typhosus* and the cholera vibrio. Park (2) recorded a decrease in the number of organisms in milk stored at 42°F. for 24 hours, a moderate increase being found when the sample was kept at 50°F. The inhibitory property became less efficient when the milk was kept at room temperature. Rosenau and McCoy (3) likewise studied the phenomenon and concluded that there is diminution in the number of organisms during the first 8 or 10 hours of incubation with a rapid growth thereafter. The action was more prolonged but less intense at 15°C. Jones and Little (4) working with the mastitis streptococcus always recorded definite inhibition during the first 4 hours of incubation and frequently multiplication did not take place during 6 or 8 hours.

Many views have been advanced in explanation of the phenonenon. Most workers favor the opinion that the milk substance is identical with blood alexin and is directly derived from the blood. On the other hand Rosenau and McCoy suggest that the lower counts after incubation in raw milk are explicable on the ground of agglutination and he infers that phagocytosis by leucocytes contained in milk may be in part responsible for the decrease.

Stocking (5) suggests that the lack of adaptation for growth in milk of the organisms employed in the experiments may be responsible for the phenomenon. Others believe that milk contains a definite bactericidal substance. This view may be said to be supported by Heinemann (6), Chambers (7), Hanssen (8), and Jones and Little. Hanssen explains the bacterial growth-inhibitory principle on the basis of the presence of oxidizing enzymes which originate in the food and reach the udder from the circulation. Jones and Little regard the substance as one resembling alexin but originating in the udder and differing from blood alexin.

From the work of others and previous work done in this laboratory sufficient evidence exists that there is in cow's milk a substance which is capable of restraining the growth of certain bacteria for definite periods. Little is known concerning the properties of the growth-inhibitory principle. Heinemann showed that it was destroyed when boiled or heated to 60°C. for 30 minutes. Chambers noted destruction at 80° or 90°C. for 2 minutes. Hanssen records that milk heated at 63°C. for 20 minutes and 70°C. for 15 minutes still retained its inhibitory activity although 75°C. for 15 minutes inactivated the substance. Jones and Little found that 62°C. for 20 minutes failed to appreciably affect the substance although 65° or 70°C. for like periods slightly altered its effectiveness; 80°C. for the same period, or boiling for 5 minutes completely inactivated it. They also showed that whey from milk coagulated by rennet contained the inhibitory principle in practically the same concentration as the original milk.

With the aim of obtaining more information about the principle a number of observations were made.

Method.

Since the methods used in the previous work had proved satisfactory the same general procedure was adopted. The milk was drawn directly from the cleansed udder, chilled, centrifuged at high speed and thus largely freed of fat, and heated at 58°C. for 20 minutes. Heating is usually advisable to rid the milk of organisms originating in the udder. After chilling it was distributed in amounts of 1 cc. into sterile agglutination tubes containing a glass bead. The tubes were then inoculated with a standard loop of 16 hour broth culture, diluted 1:200, of the non-hemolytic mastitis streptococcus, and incubated. All tubes were shaken at half-hourly intervals during the observations. For control purposes a portion of the milk or whey was boiled for 5 minutes and distributed and inoculated in the same manner. The contents of each tube was plated after definite intervals with 10 cc. of 2 per cent agar prepared from veal infusion. The plate cultures were incubated for 24 hours at 38°C. and the colonies counted.

Time of Maximum Concentration.

It is known that the concentration of the substance in the milk of young cows may be as great as in older cows. However, it is not known how soon after parturition it reaches its maximum in the secretion. In answer to this question the following observation is cited.

Experiment 1.—The colostrum and milk from cows were tested. A sample was obtained daily and refrigerated at 2–3°C. until three were on hand. They were then tested. The milk and colostrum in this experiment were heated at 60°C. for 20 minutes before they were inoculated. The protocol in Table I represents the findings in one instance.

TABLE I.

The Effect of Colostrum and Milk during Early Lactation on the Mastitis Streptococcus.

	Streptococci present after incubation at 38°C.				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Day of parturition	1,152	1,404	11,456	Innumerable	Innumerable
" after "	960	768	1,536	15,296	92,160
2 days after parturition	960	896	7,168	69,220	Innumerable
Control, 3 samples combined and boiled	1,218	4,564	Innumerable	Innumerable	"
3 days after parturition	1,024	832	2,880	10,816	40,896
4 " " "	1,024	896	1,472	72,232	Innumerable
5 " " "	960	896	7,168	69,220	"
Control, 3 samples combined and boiled	1,218	6,592	Innumerable	Innumerable	"
6 days after parturition	1,088	1,024	2,368	9,408	34,560
7 " " "	1,216	894	1,152	3,392	25,792
8 " " "	1,152	1,216	960	2,048	28,800
Control, 3 samples combined and boiled	1,344	3,860	51,840	Innumerable	Innumerable

From the protocol given in Table I it is apparent that the inhibitory principle is present in the colostrum of the 1st day but is not quite as effective as after a few days. This is not surprising since colostrum is largely an accumulated product composed to a considerable extent of blood serum proteins. The inhibitory action of the blood derivative would be inactivated at the temperature (60°C.) to which the colostrum and milk were subjected before the tests. During the first 4 or 5 days the concentration in the secretion of the inhibitory principles

is more or less variable. After this time the results are more uniform. From the findings at later periods, not recorded in the protocol given, it is certain that the principle is present in about its maximum activity after the 6th or 7th day.

Distribution in the Quarters of the Udder.

It seems logical to assume that the amount of the inhibitory principle would be relatively uniform in the secretion from the various

TABLE II.

The Effect of Milk from Various Quarters on the Growth of the Mastitis Streptococcus.

		Streptococci present				
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Cow 82	Right fore quarter	576	704	704	1,152	37,440
	Left " "	704	768	640	1,792	57,600
	Right hind "	640	512	768	576	15,552
	Left " "	640	640	5,632	Innumerable	Innumerable
" 07	Right fore "	704	704	768	1,600	72,000
	Left " "	576	704	2,112	14,400	Innumerable
	Right hind "	704	576	1,088	3,136	86,400
	Left " "	576	576	2,048	57,600	Innumerable
All samples combined and boiled		640	5,312	115,200	Innumerable	"

quarters. That this is not altogether true is evident from the next experiment.

Experiment 2.—The milk from each quarter of two cows was drawn into separate bottles. It was chilled, centrifuged to free it from fat, and heated at 60°C. for 20 minutes; then distributed and inoculated with the usual amount of dilute broth culture. Plate cultures were prepared as usual and the plates counted after suitable incubation. The results are given in Table II.

From the evidence submitted in Table II it is clear that the concentration of the inhibitory substance in the secretion from various quarters varies considerably. In the case of Cow 82 milk from the right hind quarter completely inhibited growth during the first 6

hours and considerable inhibition was noted during 8 hours. The milk from the left hind quarter inhibited during the first 2 hours but not thereafter. That from the other quarters was more efficient in this regard but not equal to that obtained from the right hind quarter. The same is true of the milk of Cow 07, the milk from the right fore quarter being more inhibitory than that from the others. It would appear that the milk from the right half of the udder contained more of the principle than that from the left. The secretion from four other cows showed similar variations. One might suppose the difference to be due to such factors as a more liberal inflow of blood serum to certain quarters or to an expenditure of the principle upon bacteria within the udder. But when the serum content of the milk specimen is measured by serum precipitin marked differences are not apparent. Furthermore the milk from a quarter invaded with streptococci may be equally as efficient in inhibiting growth under experimental conditions as that from uninvaded quarters.

Reactivation of the Principle.

If the inhibitory principle in milk is of amboceptor-complement nature, then it should be possible to inactivate it by heat and restore the activity by the addition of a little fresh milk. In order to test this point the following experiment was devised.

Experiment 3.—Milk from a single cow was obtained as usual. After freeing of fat, it was distributed in sterile tubes in amounts of 9 cc., and all tubes heated at 58°C. for 20 minutes. After chilling, two tubes were heated at 60°C. for 2½ hours, two others at 80°C. for 20 minutes, and a fifth was boiled for 5 minutes. 1 cc. of the milk heated at 58°C. for 20 minutes was added to the contents of one tube of milk which had been heated at 60°C. for 2½ hours, and a similar amount added to one of the tubes heated for 20 minutes at 80°C. The various portions were distributed in the small tubes and inoculated and tested as usual. The complete series then comprised milk heated at 58°C. for 20 minutes; two lots of milk heated for 2½ hours at 60°C., to one of which active milk was added; two lots of milk heated at 80°C., one of which was activated; and the boiled milk. The results of the tests are given in Table III.

The experiment was repeated with similar results. It is evident that 60°C. for 2½ hours does not completely inactivate the milk. The effect of adding the active milk is readily apparent but cannot be

regarded as reactivation since the substance in the active milk, combined with that still left after heating at 60°C. for 2½ hours, would be sufficient to give considerable inhibition. The results with the series which were heated at 80°C. are in agreement with this view. In my hands this temperature has been the lowest at which the principle becomes completely inactive, yet the addition of active milk restores to only a slight degree the inhibitory effect. In other experiments it has been possible to show that as little as 10 or 20 per cent of fresh milk added to boiled milk will influence the multiplication

TABLE III.

Experiment on the Reactivation of Heated Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk heated at 58°C. for 20 min.	832	576	512	640	17,280
Milk heated at 60°C. for 2½ hrs.	640	576	9,792	72,000	Innumerable
Milk heated at 60°C. for 2½ hrs. + 1/10 volume of 58°C. milk	704	576	4,032	21,880	"
Milk heated at 80°C. for 20 min.	768	17,280	Innumerable	Innumerable	"
Milk heated at 80°C. for 20 min. + 1/10 volume 58°C. milk	704	14,400	"	"	"
Boiled milk	640	23,000	"	"	"

during the first 2 hours. It seems reasonable to assume then that the substance in milk is not inactivated by heat in the sense that complement is, but that heating sufficiently to impair the inhibitory action of the milk results in actual destruction.

Filtrability of the Principle.

Is it possible to pass the inhibitory principle through filters that hold back the formed elements of the milk? From previous work it is known that whey obtained by coagulation with rennet contains the

inhibitory substance. Since whey is readily filtered it was used in the following experiment.

Experiment 4.—Samples of milk from two cows, obtained as usual, were freed of fat, mixed, and heated at 58°C. for 20 minutes. To each 150 cc. of milk, 2.5 cc. of rennet solution (1 rennet tablet dissolved in 10 cc. of 0.85 per cent NaCl solution and passed through Berkefeld candle V) was added, and after suitable incubation the whey was collected and stored in the refrigerator at 3°C. overnight. Next morning 30 cc. portions were passed rapidly through Berkefeld candles V, N, and W under a pressure of 58 to 60 mm. The unfiltered and filtered portions and whey boiled for 5 minutes were then distributed and inoculated as usual. The results of the test are given in Table IV.

TABLE IV.

The Effect of Filtration on the Inhibitory Principle.

Whey	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Untreated	1,984	1,728	1,792	1,792	51,860
Filtered through:					
Berkefeld V	2,176	1,792	1,856	2,048	12,672
" N	2,176	2,688	11,520	Innumerable	Innumerable
" W	2,026	5,568	115,200	"	"
Boiled	2,026	5,204	Innumerable	"	"

The bactericidal principle contained in whey readily passes through the pores of a Berkefeld candle V, and its activity is not appreciably impaired. A filter of N fineness withholds most of it, inhibition being noted only during the first 2 hours. The W candle takes out almost all since whey passed through this filter behaves in general like boiled whey.

The retention of the principle by filters can conceivably be due to adsorption. It is known that the removal of casein from milk during rennet coagulation fails to remove the inhibitory substance from the whey. The agent then is not readily adsorbed by the casein. The same holds true for the fat. The results of some experiments with other adsorbents are worthy of record.

Experiment 5.—Finely ground kieselguhr, kaolin, bolus alba, and animal charcoal were washed repeatedly in distilled water and dried; then weighed in amounts

of 1 and 2.5 gm. and sterilized in 50 cc. centrifuge tubes. Milk from two cows was freed of fat and heated at 58°C. for 20 minutes. To specified amounts of the adsorbents, 10 cc. of milk was added and the tubes shaken vigorously. The tubes were refrigerated 1 hour, again shaken, and then centrifuged for 10 minutes. The supernatant fluids were distributed as usual, inoculated, and tested. Inasmuch as milk mixed with 2.5 gm. of adsorbent behaved in a manner identical with that containing only 1 gm., the results of adsorption with the larger amount only are given in Table V.

The data given in Table V are derived from two separate sets of observations. The tests in which kieselguhr and kaolin were used were done in duplicate with the milk from two cows, the results of

TABLE V.

The Effect of Adsorbents on the Bactericidal Property of Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk	704	512	576	576	576
" + kieselguhr	604	462	512	384	576
" + kaolin	704	576	512	512	512
Boiled milk	640	5,440	66,240	Innumerable	Innumerable
Milk	896	640	512	704	640
" + bolus alba	832	768	768	640	704
" + animal charcoal	832	1,024	37,440	Innumerable	Innumerable
Boiled milk	768	8,640	86,400	"	"

only one series being here given. The bolus alba and animal charcoal tests were made later and were likewise done in duplicate. For this reason control findings of two lots of untreated and two lots of boiled milk are given. All the adsorbents used are negatively charged. Those that failed to adsorb the substance are slightly acid (pH 6.2 to 6.5). The animal charcoal had a pH of 7.2.

It can be said that the inhibitory principle is not adsorbed by casein, fat, kieselguhr, kaolin, or bolus alba, although considerable is taken out by animal charcoal. In this connection it is of interest to point out that blood complement is removed by kieselguhr.

The Effect of Desiccation.

A further series of experiments was undertaken to determine whether the inhibitory principle would withstand desiccation and, for the purpose, in addition to the material prepared in the laboratory commercial dried milks were employed. Several methods were employed for desiccation. Whey or milk was placed in thin collodion membranes and hung in a current of warm air. Dr. Henry Simms of this Department dried certain material by means of low pressure distillation. But the most efficient method was *in vacuo* over sulfuric acid in the refrigerator. This last method usually took 6 or 7 days. The dried material went into solution readily.

TABLE VI.

The Presence of the Bactericidal Substance in Commercial Dried Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk powder A.....	768	8,640	72,000	Innumerable	Innumerable
" " " boiled 5 min.....	704	2,304	72,000	"	"
" " B.....	832	576	704	704	57,600
" " " boiled 5 min.....	640	2,432	46,080	Innumerable	Innumerable

Experiment 6.—13 gm. of two brands of commercial milk powder was dissolved in 87 cc. of sterile distilled water. The milk was then freed of fat by centrifugation and both products heated at 58°C. for 20 minutes, a necessary precaution since both contained organisms. They were then distributed, inoculated, and tested as usual. The results are given in Table VI.

The differences in the concentration of the principle in the two samples are sharp. Product A was as good a culture medium when heated at 58°C. as it was when boiled. It appears that the principle was completely destroyed during the drying process. Product B had a well defined bacterial inhibitory action which was destroyed by boiling.

With milk or whey dried in the laboratory the results showed that the inhibitory principle would withstand drying but they were not as

striking as with commercial product B. This is brought out in Experiment 7.

Experiment 7.—Fat-free milk heated at 58°C. for 20 minutes was distributed in thin layers in sterile wide mouth bottles and desiccated over H_2SO_4 *in vacuo*. Low pressure was maintained by the use of an oil pump twice daily. Between evacuations the jars were stored at a temperature of 3° or 4°C. Under these conditions 7 days was required for complete drying. The residue before use was dissolved in sterile distilled water and the product tested in the usual manner. Some of the material was tested as soon as possible and the remainder in the form of dry residue stored in the room and dissolved and tested 18 days later. The results are given in Table VII.

TABLE VII.

The Effect of Desiccation on the Bactericidal Principle.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Sample tested shortly after desiccation	640	896	576	4,316	72,000
The same boiled for 5 min.	768	6,912	86,400	Innumerable	Innumerable
Sample after 18 days storage in the room	256	320	9,472	51,840	"
The same boiled for 5 min.	320	9,216	72,000	Innumerable	"

The milk used in the experiment would completely inhibit growth for 6 hours and markedly influence it for 8 hours, but the dried preparation was not quite as efficient in this regard. Storage of the dried material at room temperature led to further inactivation. Desiccation by evaporation through collodion membranes over $CaCl_2$, or by low pressure distillation, led to no better results. Some of the substance always remained in the residue but never to the same concentration as in the original material and its activity deteriorated on standing.

If it were possible to obtain the substance in a concentrated form, considerably more could be learned of its properties. When one or two volumes of alcohol is added to whey and the supernatant and residue completely freed of alcohol by low pressure distillation, both

products, added to boiled milk, fail to inhibit growth. One may assume then that alcohol destroys the principle.

It was hoped that the agent adsorbed on particles of charcoal could be redissolved in more concentrated form. Several experiments were made in which milk was adsorbed with charcoal and the charcoal mixed with dilute acids and alkali, dilute phosphate solutions, physiological sodium chloride solution, and distilled water, and permitted to stand for several days in the refrigerator, the supernatant solutions finally being added to boiled milk and tested. It was possible to obtain a little inhibition with the extracts of weak alkali, phosphate, and sodium chloride, but the results obtained were not encouraging enough to warrant further work.

DISCUSSION.

Substances contained in a complex fluid like milk may be attached to or so closely associated with some of the other components that their true behavior may be masked. Granting this the inhibitory principle must yet be regarded as a definite constituent of cow's milk which is secreted with the colostrum and early milk. Other observations not reported in this paper indicate that the substance is present in the milk throughout the lactation period. It is a characteristic ingredient of the milk, being present, according to our experience, in the secretion of all cows though it is true that its concentration varies in different animals and may even vary in the milk from different quarters of the same udder.

It has already been stated that several views are prevalent regarding the nature of the principle. Stocking's contention that milk affords an improper medium for the test organisms is open to question in view of the fact that boiling milk for 5 minutes renders it an admirable medium. Further the organism employed throughout the test is an udder inhabitant and so far as can be learned exists only in milk. The action of phagocytes is ruled out in milk that is heated at 58° or 60°C. for 20 minutes and further by the results of the filtration experiments. Agglutination of the streptococcus was not found on microscopic examination. Some have supposed the principle to be blood alexin, others that it is a ferment derived from food, and yet others that it is an alexin-like substance originating in the udder.

The third view would seem to be the most reasonable. It is known that blood alexin loses its antibacterial action when diluted, and it must be greatly diluted in milk in the light of the fact that serum globulin is present in milk in small quantities only. The milk substance is much more resistant to heat than blood alexin. Unlike alexin once its activity is impaired by heat it cannot be reactivated by active milk. It is not adsorbed by the same class of adsorbents. Colostrum, which contains a large proportion of blood serum, inhibits bacterial growth even when heated to 60°C., a temperature sufficient to inactivate blood alexin.

The inhibitory substance in milk may serve to protect the udder from the growth of many types of organisms. The usual period of bacterial inhibition for which it is responsible corresponds roughly to the period between feedings by the calf which would empty the udder under natural conditions.

SUMMARY.

Certain of the properties of the bacterial agent in milk have been studied. The substance is present in the colostrum and milk of the first few days of lactation as well as later. Its concentration varies in the secretion from various quarters of the same cow. Its activity is diminished by heat and cannot be restored again by the addition of active milk. The principle is present in whey and readily passes through the coarsest Berkefeld filter although a considerable portion is retained by N candles. The finest filter (W) completely retains it. It is adsorbed by animal charcoal but not by kaolin, kieselguhr, or bolus alba. It can be desiccated and its presence has been demonstrated in one brand of dried milk.

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BACTERIAL VARIATION IN CULTURES OF FRIEDLÄNDER'S BACILLUS.

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Plates 36 and 37.

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During the course of studies on the biological and immunological properties of Friedländer's bacillus, at least three sharply defined types were found (1) to exist among different strains of the organism. The types were designated A, B, C, and into one Group, X, were placed several heterogeneous strains. That Friedländer's bacillus, moreover, possesses at least two different cellular constituents both of which are important in the antigenicity of the organism was recorded in later communications (2, 3). One of these is now known to be polysaccharide and is chemically different for each of the fixed types (4, 5). The differential specificity of the types appears to depend on the polysaccharides, a class of substances which are analogous to the soluble specific substance of Pneumococcus, originally described by Dochez and Avery (6). The second substance is protein in nature and regardless of type derivation exhibits the undifferentiated characteristics of the species. To this extent, Friedländer's bacillus possesses an antigenic complex which is analogous to that of Pneumococcus (7) as already described in papers from this laboratory.

It has been pointed out by other investigators (8–14) as well as ourselves that there are certain conditions which induce in Friedländer's bacillus the development of variants, a phenomenon which is now considered as bacterial dissociation.[1] The typical colonies of Friedländer's bacillus are now identified as smooth (S) and the variant colonies as rough (R), the organisms in each instance differing not only morphologically, but biologically and antigenically. The most salient

[1] For an analytic and critical review of the literature on microbic dissociation, the reader is referred to Hadley's monograph in the *J. Infect. Dis.*, 1927, xl, 1.

differences are (2) that the S cells form colonies with a smooth surface; they are virulent, encapsulated, and type-specific; and they produce the soluble specific substance. On the other hand, the R cells form colonies with a rough surface; they are avirulent, capsule-free, and not type-specific, but species-specific; and they do not elaborate the soluble specific substance. Furthermore, sera prepared by immunization with S cells are type-specific and passively protect against fatal infection by bacilli of the same type, while anti-R sera are not type-specific and possess no demonstrable protective properties for infected mice.

Further study has since disclosed that there exist among the R variants of Friedländer's bacillus additional dissociates which present definite differences in morphology and antigenicity. Moreover, observations have been made upon some of the conditions conducive to variation, the possible reversibility of the variants to their antecedent forms, and the occurrence of variants in human infection. The present communication comprises a report of these studies.

That several morphological forms or intermediates exist between the extremes of the R and S varieties of the Friedländer's bacillus has been reported by Toenniessen, Baerthlein, and Hadley. The work of the two former writers antedated the use of the present nomenclature.

EXPERIMENTAL.

Without entering into a discussion as to what constitutes a Friedländer's bacillus, the S strains studied in this investigation were all members of the *Encapsulatus* group as determined by cultural and staining reactions. They were all Gram-negative, encapsulated bacilli, which were virulent for mice and showed great variation in sugar fermentation reactions. The strain "SC" (Type A) which was most studied in the present investigation, was isolated from the blood of a fatal case of pneumonia in man. It fermented without gas, dextrose, sucrose, maltose, mannitol, and lactose.

The R strains employed in the previous study were derived by consecutive transfer of S cells in broth to which was added a concentration of 10 per cent homologous immune serum. Plate cultures were made after each transplant until R colonies were grossly visible. In the earlier studies, the R colonies were not examined for structural differences, but at a later period, however, three different forms of R colonies were recognized.

The characteristic colonies of the S and R variety have been observed with each of the serological types of Friedländer's bacillus. A detailed

study, however, has been made of those derived from Type A only, and the data, unless otherwise stated, refer solely to this type.

I. Forms of Variants Encountered.

In addition to the S variety, three different forms of colonies have been recognized. For the sake of convenience and clarity of expression these three R variants may be designated as R1, R2, and R3. Further than gross appearance the R3 colony was not studied, because it was found only rarely and sporadically in mass cultures of R cells and particularly because it was extremely unstable so that the organisms were never obtained in pure culture. The R3 colony was of the "phantom" description, escaping notice when viewed by transmitted light on account of its marked transparency. By reflected light, with most of the light obscured, it was seen as a transparent colony with a smooth surface and an annular margin which appeared slightly raised and fringed.

(*a*) *Morphological Differences.*—The S colony, examined by transmitted light after 15–18 hours of growth, appears opaque, white, sharply circumscribed, markedly convex, homogeneous, and circular (see Fig. 1). When seeded heavily the colonies coalesce, and this mucoid coalescent growth is typically characteristic of S organisms. By reflected light the surface of the S colony is glistening and smooth and reflects sharply and accurately the image of objects within focal distance. The colony growth is mucoid and tenacious in consistency, and is elastic to the touch of a needle. The S organism is Gram-negative, and encapsulated (Figs. 2 and 3). The rods are short and thick and most commonly occur in either single or in diplo forms.

The R1 colony by transmitted light appears transparent, pale yellow or tan in color with a slightly indented border; it is flat with a distinct central papilla that becomes more prominent with age; it is not homogeneous, and it is more or less circular in shape (see Fig. 1). By reflected light, the surface appears uneven and glossy and reflects images definitely but in a distorted fashion. Due to the manner in which the light is reflected the center of the colony seems to be raised into a small cone. The colony growth is more discrete, never as tenacious as S, and is readily picked with a needle. Stained preparations exhibits a short, slender, almost coccoid bacillus, which is Gram-

negative and unencapsulated. The forms are often so small as to suggest morphologically *B. influenzæ* (Figs. 4 and 5).

The R2 colony by transmitted light appears transparent, pale yellow or tan in color, with wavy margins; it is flat, not homogeneous, but appears matted simulating a tuft of cotton (see Fig. 1). The shape of the colony varies, but in general it is circular. By transmitted light, the surface markings suggest an oyster shell, *i.e.* irregular concentric rings with rough surface, and the reflection of images is always distorted. The colony growth is not confluent, mucoid, or tenacious and is picked readily. Stained preparations show long, slender, Gram-negative bacilli which, in young cultures, often occur in long wavy chains or threads. The organism is not encapsulated (Figs. 6 and 7).

The size of the S and the R colonies varies considerably, but in a general way, the R2 colonies are the largest, the S next in size, and the R1 are the smallest. Morphologically R2 bacillus is the longest while the R1 is the shortest. The S, on the other hand, shows the greatest dimension in breadth.

Figs. 1 to 7 demonstrate the relative differences of S, R1, and R2, both in colony formation and in microscopical appearance.

(*b*) *Biological Differences.*—Culturally, the S and R forms exhibit as striking differences as they do morphologically. The growth of the S cells in fluid media is viscous and accompanied by the production of soluble specific substance. The S form is always encapsulated and of marked virulence for mice, the intraperitoneal injection of 1/10 millionth cc. of a young culture (6–8 hours) causing death within 24–48 hours. In such instances, the peritoneal exudate is viscous, contains relatively few leucocytes which are frequently surrounded by a clear zone separating them from the bacilli, and phagocytosis of the organism has never been observed in normal animals.

The growth of R1 and R2 in fluid media is diffuse, non-viscous, and not accompanied by the elaboration of specific soluble substance. The organisms are not encapsulated and the virulence of both forms is extremely low, since doses as large as 0.5 cc. of a young culture frequently fail to cause fatal infection in mice. The peritoneal exudate following the injection of R1 or R2 is not viscous and contains numerous leucocytes which are able apparently to phagocyte the bacteria.

The fermentation reactions of the three strains were also studied.

The carbohydrates tested were dextrose, lactose, sucrose, maltose, and mannitol. As will be seen in Table I, both the S and the R strains fermented each of the sugars within the first 24 hours of growth, except that in the case of R1, the fermentation of lactose on the two occasions tested did not occur until the 6th day.

(*c*) *Antigenic Differences.*—It has already been shown in a previous communication (3) that S strains of Friedländer's bacillus induce in rabbits the formation of antibodies which agglutinate type-specifically, precipitate the corresponding specific soluble substance, and protect white mice against infection by strains of the same type. Anti-S sera may in addition contain species-specific antibody depending upon

TABLE I.

Biological Reactions of S, R1, and R2 Strains of Friedländer's Bacillus (Type A).*

Strain	Capsule	Specific soluble substance	Virulence	Phagocytosis	Fermentation reactions				
					Lactose	Dextrose	Sucrose	Mannitol	Maltose
S	+	+	+	−	+	+	+	+	+
R1	−	−	−	+	+x	+	+	+	+
R2	−	−	−	+	+	+	+	+	+

* The S strain was isolated from the blood of a fatal case of pneumonia in man.

+ indicates presence.

− indicates absence.

x, fermentation was delayed to 6th day, whereas all the other fermentations occurred within 24 hours.

the duration and intensity of the immunization. Consequently, anti-S sera may cause agglutination of R cells. Anti-R sera, on the other hand, are lacking in antibodies associated with type specificity and protection, and contain only the common group antibody which reacts with R organisms derived from any of the serological types. The R strains employed in these reactions, however, were mass R cultures and represented one colony arising from the continued growth of an S strain in homologous anti-S serum.

The antigenic character of R1 and R2 was correlated with that of the original S strain from which they had arisen. Antisera were prepared by the intravenous injection of rabbits with heat-killed suspensions (2) of S, R1, and R2, respectively. With the resulting immune

sera, it was established that S was agglutinated in anti-S sera, but not in either R1, or R2, antisera, as is brought out in Table II. R1 and R2 were agglutinated in anti-S serum to a slight extent, the reaction appearing granular in contradistinction to the disc reaction occurring with S in anti-S sera (2).

TABLE II.

Cross-Agglutination Reactions with S, R1, and R2 Strains of Friedländer's Bacillus (Type A).

Strain	Immune serum					
	Anti-S		Anti-R1		Anti-R2	
	1:5	1:10	1:5	1:10	1:5	1:10
S	++++	++++	−	−	−	−
R1	++	+	++++	++++	++++	++++
R2	++	+	++++	++++	++++	++++

In this and following tables ++++ indicates complete agglutination with flocculent precipitate and clear supernatant; +++, almost complete, supernatant clouded; ++, marked agglutination; +, slight agglutination; −, no agglutination.

TABLE III.

Cross-Agglutination Reactions with R1 and R2 Strains of Friedländer's Bacillus (Type A).

Immune serum	Strain	Final dilution of serum									
		5	10	20	40	80	160	320	640	1280	2560
Anti-R1	R1	++++	++++	++++	++++	++++	+++	+++	++	+	−
	R2	++++	++++	++++	++++	+++	+++	++	+	−	−
Anti-R2	R1	++++	++++	++++	++++	+++	+++	++	++	+	+
	R2	++++	++++	++++	++++	++++	++++	+++	+++	++	+

The immune serum of R1 was found to agglutinate both R1 and R2 to about the same extent, and conversely R2 antiserum caused an equally good agglutination of both strains. Agglutination was the typical R variety of granular sedimentation which breaks up readily upon agitation. The antigenic similarity evidenced by the agglutina-

tion reaction was further studied by means of agglutinin adsorption. R1 and R2 immune sera were adsorbed with both strains, and then tested for the presence or absence of agglutinins. The results of the experiments can be summarized briefly: Each strain (R1 and R2) adsorbs from the homologous antiserum agglutinins for both homologous and heterologous organisms; from the heterologous serum, however, antibody is removed only for the strain employed in the adsorption. In other words, R1 and R2 possess mutual agglutinating characters, but not complete, mutual adsorptive properties, as deter-

TABLE IV.

Agglutinin Adsorption Reaction.

Results of Agglutination with R1 and R2 Serum after Adsorption with R1 and R2 Strains.

Immune serum	Adsorbed with	Antigen	Final dilution of serum							
			20	40	80	160	320	640	1280	2560
Anti-R1	R1	R1	−	−	−	−	−	−	−	−
		R2	−	−	−	−	−	−	−	−
	R2	R1	++++	++++	++++	+++	++	++	+	−
		R2	−	−	−	−	−	−	−	−
Anti-R2	R1	R1	−	−	−	−	−	−	−	−
		R2	++++	++++	++++	++++	+++	+++	++	+
	R2	R1	−	−	−	−	−	−	−	−
		R2	−	−	−	−	−	−	−	−

mined by reciprocal adsorption. The data of these experiments are presented in Table IV.

As was to be expected from previous results (2) neither R1 nor R2 immune sera caused agglutination of the S strain from which they originated, nor did they cause precipitation of specific soluble substance, nor passively confer immunity upon mice infected with the antecedent S strain.

II. The Reversibility of R to S.

The R strains studied in this investigation have been remarkably stable during the 2 years they have been under observation. Since

the permanency of R becomes of paramount importance when viewed in terms of the problems of infection and epidemiology, experiments were planned to determine the reversibility of R to S. The older literature particularly with other species offers some evidence in favor of reversibility, but the objection has been raised that mass cultures were studied instead of pure line strains. More recently, however, it has been shown unimpeachably that single cell cultures of R may be caused to revert to S under proper cultural conditions. Thus Jordan (15) and Soule (16) showed interconvertibility of *B. paratyphosus* B, Levinthal (17), and Dawson and Avery (18), of Pneumococcus, Soule (19), of *B. subtilis*.

In the present study of reversibility, single cell strains were obtained by the technique of Avery and Leland (20). Since all experiments with pure line strains uniformly failed to bring about reversion, mass R cultures were studied instead, because such cultures might contain individual organisms with greater potentialities for reversion than the single R cells chosen at random. The observations were made with cultures derived from each of the three serological types. The methods adopted for reversion were (1) rapid transfer through meat infusion broth, (2) rapid transfer through dextrose broth, (3) growth in the supernatant culture fluid of the parent S strains, (4) growth in anti-R sera, (5) passage through normal white mice both before and after preliminary transfer through anti-R sera. The greater part of the experiments were carried out before our recognition of the two distinct forms of R variants and the mass cultures studied may have been mixtures of both forms. The results obtained with each method are briefly summarized below.

(*1*) *Rapid Transfer through Meat Infusion Broth.*—One strain each of both mass and single cell R cultures, derived from the three serologically different types, was carried through 90 transfers in meat infusion broth. Transplants were made two or three times daily and from time to time plates were streaked to examine colony formation and the cultures were tested for agglutination by the homologous anti-S serum. The reversion of R to S was not observed by this method.

(*2*) *Rapid Transfer through Dextrose Broth.*—It had been noted earlier in the study that a number of R strains which fermented

dextrose, grew in this medium in conglomerate clumps or masses strongly suggestive of a thread reaction. It is interesting to note in this connection that this phenomenon was never noticed in acid fermentation by S strains. Two mass cultures of R forms derived from a single colony were transplanted once daily in 1 per cent dextrose broth for 35 transfers. By the sixth subculture, clumped growth no longer occurred, although dextrose was still fermented. No evidence, however, was obtained of reversion.

(*3*) *Growth in the Supernatant of the Parent S Strain.*—18 hour broth cultures of S strains were centrifuged and the supernatant was withdrawn and rendered sterile by heating at 56°C. for 30 minutes. This was added in 10 per cent concentration to infusion broth alone and to infusion broth containing 10 per cent anti-R serum. In such media mass R cultures were transplanted twice daily for 90 transfers. At no time during the period of observation was reversion encountered.

(*4*) *Growth in Anti-R Sera.*—Both mass and single cell R strains derived from each of the serological types were carried twice daily through 10 per cent anti-R serum broth for 90 generations. The anti-R serum used in the different experiments was both homologous and heterologous and later, mass cultures of R organisms derived from Type A were carried through 40 transplants in 1 per cent and 5 per cent anti-R serum broth. In the earlier transplants growth always appeared in thread formation, that is, clumped in the bottom of the tube with a clear supernatant fluid. After 20 to 40 or more transplants this reaction disappeared and growth was uniformly diffused. Although in some instances the colony growth seemed to be somewhat less rough, nevertheless, reversion did not occur.

(*5*) *Passage through Normal White Mice.*—Each mass culture from the preceding experiment (*i.e.* after 90 transplants in anti-R serum broth) was passed through normal mice by intraperitoneal injection. As controls, two other R strains—one derived from Type A and the other from Type B—were passed through mice without preliminary growth in anti-R sera. Mice were injected with large amounts of young R cultures and the peritoneal washings reinjected into other normal mice. This was carried out with each strain through a series of 22 mice but in no instance did reversion occur.

(6) *Experiment with R1 and R2.*—The foregoing experiments on the reversion of R to S were carried out as stated with either mass cultures or pure line strains without regard to the particular form of the R variant studied. It seemed possible, however, that the question might now be answered more accurately and completely by a study of the two well defined variant forms R1 and R2. Cultures of each variety, therefore, were transferred twice daily in broth to which had been added in one series 10 per cent homologous immune serum, and in another series 10 per cent heterologous immune serum. The strains were grown in this way for 60 transplants and after 30 to 38 transfers the thread reaction had disappeared. Under these conditions it was possible to induce R1 to change to R2 but reversion of either variant to the S type was not observed.

In summarizing, then, the results of the study of reversion, it may be stated that none of the methods employed, succeeded in bringing R forms back to the S type.

This does not mean, however, that R forms are irreversible, but that under the conditions stated, the methods employed were not adequate to effect the change.

III. Some Incitants to Variation.

(a) *Experimental Derivation of R Forms.*—Mass R cultures may be experimentally derived by the continued subculture of S cells in broth to which has been added homologous immune serum, R organisms gradually appear as the S forms disappear. It is an old observation among earlier workers, however, that Friedländer's bacillus upon aging gives rise to variant colonies which differ strikingly in certain characters, the authors reporting on some or all of the properties of virulence, agglutination, and colony appearance. Our experience corroborates these results and included the isolation of R forms from aged colonies on plates and occasionally from broth cultures stored for several weeks, in which the change has spontaneously occurred.

(b) *Occurrence of R Forms in Disease.*—It is definitely known that R variants may be experimentally derived *in vitro* from S cells. The phenomenon of bacterial dissociation, however, would acquire greater significance if it could be demonstrated that the process actually takes place in the animal body during the course of infection. In order to

study this possibility, a survey was made of strains freshly isolated from a number of different pathological conditions and a careful search was made for the presence of R variants. In all, cultures from seventeen different sources were examined and these included seven cases of human pneumonia, one case of pneumonia in a guinea pig, two of liver abscesses in man, two of acute and fatal abscesses in guinea pigs, two of cystitis in man, one of infected antrum in man, and two cases of infected adenoid tissue. In five instances R forms were iso-

TABLE V.

The Occurrence of R Variants in Infections Associated with Friedländer's Bacillus.

Case No.	Source	Type	Presence of R
1	Sputum, Autopsy } pneumonia	A	Not found
2	Sputum—pneumonia	A	Not found
3	Sputum—pneumonia	A	Not found
4	Sputum—pneumonia	A	Not found
5	Sputum—pneumonia	A	Not found
6	Abscess—guinea pig (fatal)	A	Not found
7	Abscess—guinea pig (fatal)	A	Not found
8	Liver abscess	A	Not found
9	Adenoid tissue	A	Present
10	Adenoid tissue	A	Present
11	Autopsy—pneumonia (guinea pig)	B	Not found
12	Sputum—pneumonia	B	Not found
13	Infected antrum	C	Present
14	Sputum—pneumonia	Group X	Not found
15	Urine—cystitis	Group X	Not found
16	Urine—cystitis	Group X	Present
17	Liver abscess	Group X	Present

Except where stated, the strains were derived from human infections.

lated and in each they were present in mixtures of R and S. Since the occurrence of the two distinct variants, R1 and R2, was recognized only after this survey was completed, it is impossible to state the relative frequency of these two forms. However, of the R strains isolated, two were present with S organisms of Type A, one with those of Type C, and two others in association with S cells of Group X. Interestingly enough, the R strains were found not in acute infections but in chronic conditions. Thus R forms were present twice in cultures from adenoid

tissue, twice in cases of chronic cystitis, and once from a subacute antrum. Suggestive as the data are, no generalization, however, can be made from so few observations. The details of this study are recorded in Table V.

DISCUSSION.

The study of variation in cultures of Friedländer's bacillus reported in the present communication discloses three different forms of R variants. Two of the variants (R1 and R2) have been studied in detail, and they may be recognized grossly by colony formation or microscopcally by the size and arrangement of the individual cells. Moreover, it has been possible to differentiate the dissociates further by serological reactions. Both variants (R1 and R2) are agglutinated in antisera prepared by injection of rabbits with either strain, but they lack the capacity of complete reciprocal agglutinin adsorption. The two R strains are markedly different from their antecedent S strain in colony appearance, morphology, virulence, and antigenicity.

A number of methods have been adopted to induce reversion of R to S. Whether the technique or its application was inadequate, the results were uniformly negative. This does not imply, however, that all R forms of Friedländer's bacillus are irreversible, but that in the case of the strains studied, the proper stimulus was not supplied by the methods used. In this connection the work of Dawson and Avery (21) offers an interesting comparison. They found one R strain of Type I pneumococcus irreversible by the identical methods which caused other R strains of the same and different types to change to the S form. In the present study, R2 has been converted to R1, while, on the other hand, R1 itself has remained unchanged following numerous transplants in homologous immune serum. Conversion of R2 to R1 and the less rough appearance of R1 colonies make it not unlikely that R1 is an intermediary form between S and R2.

The spontaneous development of R variants in S cultures of Friedländer's bacillus has been found to accompany the process of aging. Growth in immune sera *in vitro* also converts the S cells into R forms. That variation, however, is more than an *in vitro* or cultural degradation gains support from the fact that R forms have been found in cultures taken directly from foci of infection in the animal body caused

by Friedländer's bacillus. It is an interesting observation that in the cases studied R variants were found only in chronic infections and always in conjunction with S forms.

CONCLUSIONS.

1. Under proper conditions mass R cultures of Friedländer's bacillus may give rise to a number of variants which are dissimilar in colony appearance and morphology. Three such forms have been described. In two varieties, differences have been observed not only in colony formation and morphology, but also in cultural and antigenic characters.

2. None of the methods employed were adequate to cause reversion of any of the R variants to the S type. Growth of the R2 variant in its own antiserum, however, induced a change to the R1 form.

3. R forms of Friedländer's bacillus may be derived from S strains by aging or by growth in anti-S serum of the homologous type.

4. R strains may be isolated in culture directly from infection. In the cases where R forms were found, S cells were also present, and the pathological condition was of a chronic nature.

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EXPLANATION OF PLATES.

PLATE 36.

FIG. 1. Plate culture of Type A, Friedländer's bacillus, taken by transmitted light. The S colony and the two variant forms, R1 and R2, are labelled. Note opacity of S and transparency of both R varieties.

FIG. 2. Smear of peritoneal exudate of mouse infected with S. Stained with Gram, × 1000. Note absence of leucocytes and presence of large capsules.

FIG. 3. Smear of S grown on agar. Gram stain, × 1000. Capsule is greatly diminished.

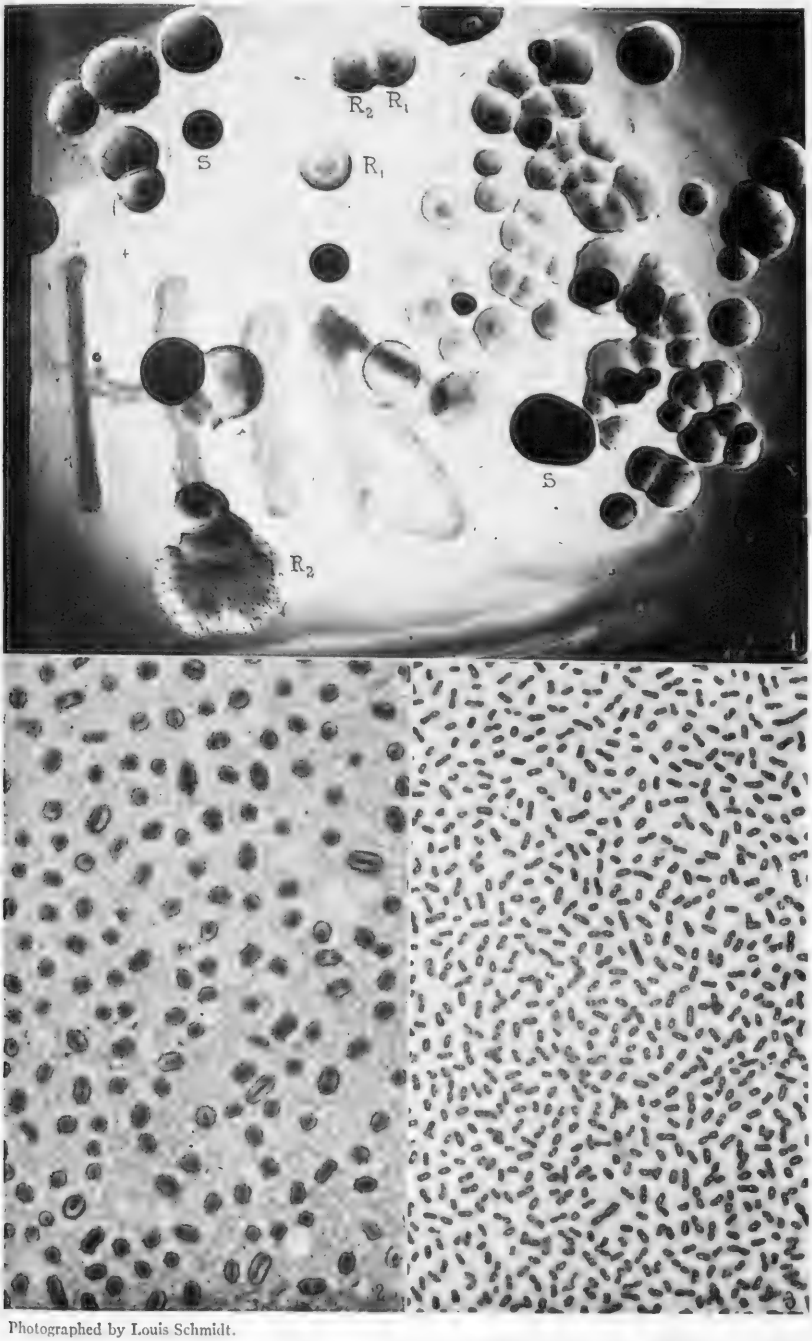
PLATE 37.

FIG. 4. Smear of peritoneal exudate of mouse injected with R1. Stained with Gram, × 1000. Note phagocytosis and lack of capsules.

FIG. 5. Smear of R1 grown on agar. Gram stain, × 1000. Note size and arrangement as contrasted with S and lack of capsules.

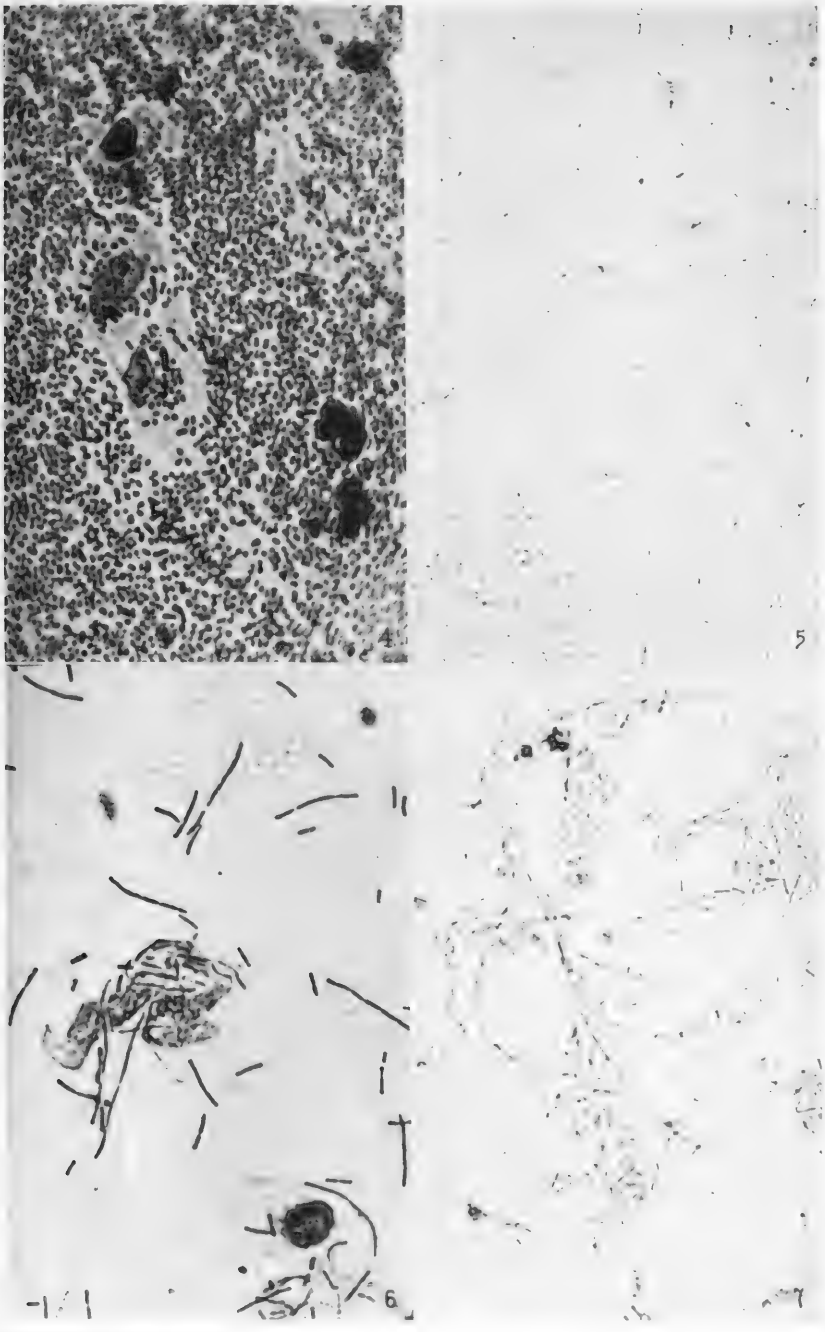
FIG. 6. Smear of peritoneal exudate of mouse injected with R2. Stained with Gram, × 1000. Note phagocytosis, lack of capsules, and length of rods.

FIG. 7 Smear of R2 grown on agar. Gram stain, × 1000. Note size and arrangement and lack of capsule.



Photographed by Louis Schmidt.

(Julianelle: Cultures of Friedländer's bacillus.)



Photographed by Louis Schmidt.

(Julianelle: Cultures of Friedländer's bacillus.)

IS THERE AN IMMUNOLOGICAL RELATIONSHIP BETWEEN HORSE SERUM AND HORSE DANDER?

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Although an antigenic relationship between horse dander and horse serum seems to have become a tacit assumption on the part of many immunologists, practically no experimental work has been aimed directly at the proof or disproof of it. The fact that a considerable proportion of asthmatics shows a cutaneous sensitiveness both to the dandruff and to the serum of a given animal species (1) has probably been responsible for this assumption, but such evidence is, of course, purely circumstantial.

Earlier Studies.

The clinical importance of horse dander as a provocative of asthma has made it the subject of much study during the last 15 or 20 years. Laboratory studies have been pursued mainly along two lines: (1) sensitization of experimental animals, usually with the object of reproducing asthmatic symptoms; (2) chemical separation of dander into its several constituent antigens.

The difficulty experienced in the sensitization of laboratory animals with horse dander has led more than one investigator to question the antigenic properties of the substance. One of these expressions of doubt bears so recent a date as 1923 (2), in spite of the considerable amount of evidence that has accumulated since horse dander was first implicated in the causation of asthma. This point has recently been reinvestigated by Longcope, O'Brien, and Perlzweig (3) and their work shows conclusively that a saline extract of horse dander is capable of sensitizing and producing anaphylactic death in guinea pigs.

Still more recently Alexander, Becke, and Holmes (4) have succeeded in sensitizing guinea pigs by exposing them to a spray of saline extract of horse dander.

Such animals showed no symptoms when again exposed after a suitable interval to this spray, but when tested by the uterine strip method in a Dale apparatus, responded with marked contraction upon the addition of dander extract to the bath.

Wodehouse (5) has made an extensive study of the separable antigens of horse dander as well as those of the hair of cats and dogs. As principal constituents of each of these he found in his weakly alkaline (N/100 KOH) extract (1) an acid-precipitable substance which he called "alkali meta-protein," (2) an "acid meta-protein," precipitated by addition of alkali to the acid filtrate from the preceding, and (3) a peptone. In addition to these he found in horse dander (4) an alcohol-soluble protein and (5) a heat-coagulable protein.

Longcope and his associates (6) have recently used more exact methods of isolation and have obtained two fractions of antigenic importance from horse dander, one of which, their protein B, makes up much the larger portion of the substances in solution. This fraction is precipitated between pH 3.2 and pH 3.8 and is probably to be identified with the "alkali meta-protein" of Wodehouse. The second fraction, their protein D, precipitates at pH 12.0, is much smaller in amount, and may possibly be the same as Wodehouse's "acid meta-protein." It is difficult to correlate these two studies exactly because the latter author did not use pH values in his description. It is further noteworthy that Longcope and his coworkers found no precipitation occurring at the isoelectric points of serum globulin or albumin.

Ratner, Jackson, and Gruehl (7) have made a very brief report which has a more direct bearing upon the results here submitted. This report states that they have obtained cross-anaphylactic reactions with horse dander and horse serum. No further details of their experimentation are given, however, except the remark that the Dale method was not relied upon as a final criterion for anaphylaxis. The writer has not encountered their further report in subsequent literature.

EXPERIMENTAL DATA.

Three routes of approach have been employed in the effort to determine whether an antigenic relationship exists between horse serum and horse dander: (1) cross-precipitation tests, rabbits being used for precipitin production; (2) cross-anaphylactic tests in guinea pigs by the uterine strip method of Dale; (3) cross-anaphylactic tests by the usual method of producing dyspnea and fatal shock in guinea pigs.

Antigens.—The dander antigens employed were prepared by various methods, but were of two principal types: (1) saline suspensions of whole dander and (2) saline extracts of dander.

In preparing the whole dander suspensions 5 gm. of the dry dander was suspended in 100 cc. of neutral 0.85 per cent NaCl by shaking with glass beads. At

first these suspensions were partially sterilized (phenolized or formalinized) to avoid severe abscess production which usually followed its intraperitoneal injection into rabbits and guinea pigs. It was found, however, that the mass of small hairs present in suspension was chiefly responsible for the abscesses. Centrifugalization removes the greater portion of hair, and abscesses were thus avoided. No difference was found in the immunizing value of the two preparations.

For the saline extract of horse dander the same proportions were used, namely, 5 gm. of dry dander per 100 cc. of neutral 0.85 per cent NaCl solution. The extract, however, had a reaction of pH 7.2 to 7.6 without buffering. A drop or two of phenol red was added to the saline to indicate the reaction. The suspension was then shaken with beads for 4 or 5 hours and centrifugalized for about an hour in order to throw down the heavier part of the material. The supernatant was then filtered either through a Berkefeld or through a Seitz filter, and was ready for use, a clear amber fluid, tinged by the phenol red. Considerable difference in antigenic value was found between the products of these two filters. This will be referred to again below.

The filtrate was found to contain as its chief antigenic constituent a protein, precipitable by dilute acetic acid, which seems to answer to the "alkali metaprotein" of Wodehouse (5) and to the protein B of Longcope, O'Brien, and Perlzweig (6). This acid-precipitable substance was obtained by adding drop by drop, stirring meanwhile, a minimal amount of N/1 acetic acid for producing maximal precipitation. Two more precipitations were done for purification. That this purified fraction is fairly efficient as an antigen was demonstrated by the production of precipitin titers as high as 1–640 and by the active sensitization of one guinea pig, tested by the uterine strip method. At least one other protein was present in horse dander, but in much smaller proportions. Its isolation was not attempted.

Both whole dander suspensions and the saline extracts elicited precipitating sera of comparatively high titers. Only the extract served, however, for the precipitation tests because of the turbidity of the suspensions.

Precipitation Tests.—Ten antisera against horse dander (eight against whole dander and two against dander extract) were produced in rabbits, their titers varying from 1–160 to 1–10,240. In six of them cross-precipitation was obtained. That is to say, these six antidander sera precipitated not only homologous antigen (dander extract), but also normal horse serum. The other four antidander sera had such low titers that cross-precipitation was not attempted.

At first the reciprocal cross-reaction was not obtained, namely, the precipitation of horse dander extract by antisera against horse serum. Four of the latter antisera, ranging in titer from 1–2,560 to 1–10,640, were titrated with Berkefeld-filtered saline extract of horse dander with

negative results. Two of these antisera, when subsequently titrated against Seitz-filtered dander extract,[1] precipitated this antigen. The other two sera had not been preserved. Three additional sera have since been produced against horse serum, however, with all of which cross-precipitation has been obtained, with the Seitz-filtered antigen. Five of these antisera against horse serum have, therefore, precipitated horse dander extract.

The accompanying protocols (Tables I and II) show how the cross-precipitation tests were carried out. Forensic precipitation proportions (0.2 cc. of undiluted antiserum plus 1.0 cc. of antigen dilution, or half these quantities) were used.

Table I records the results of the titration of an antiserum against horse dander. The first group of results represents a negative control titration of undiluted antiserum against successive dilutions of an alien serum (normal human serum). Observations were made of the ring test (after 15 minutes), the flocculation test (after $1\frac{1}{2}$ hours at 38° or 2 hours at room temperature), and the sedimentation test (after overnight ice box temperature). The second group of results represents a positive control titration of the antiserum against serial dilutions of homologous antigen (Berkefeld-filtered extract). The third group represents a cross-titration of the antiserum against serial dilutions of normal horse serum. The vertical column at the extreme right indicates the antiserum-saline control.

Table II shows the results of a titration of an antiserum against horse serum. The negative control antigen in this case was normal guinea pig serum, the positive control antigen normal horse serum, the antigen for cross-titration Seitz-filtered extract of horse dander.

The cross-titers obtained in the two cases represented are markedly

[1] No definite study was made to determine the reason for the difference in these two filtrates. It was observed, however, that the first portion of every Berkefeld filtrate was acid (to phenol red). The alkalinity of the dander extract neutralized the acidity of the filter after a few cc. of the filtrate had been delivered. The first few cc. came through the filter rapidly, but thereafter the filtrate was obtained very slowly. Considering the slight acidity required to precipitate the principal protein in horse dander extract, it seems reasonable to suppose that the acidity of the Berkefeld filter precipitated enough of the dander protein to clog the filter and thus increase its fineness. The Seitz filter, being neutral, offers no such problem.

TABLE I.

Titration Record of an Antiserum against Horse Dander.

Serum 396 (against horse dander).

t = turbidity + = flocculation, sedimentation, or positive ring test − = negative of two preceding		1/5			1/20			1/100			1/500			1/2,000			1/10,000			1/50,000			1/200,000			Anti-serum Saline control		
Negative control Serum 396 *vs.* normal human serum	Ring Flocculation Sedimentation	−	t	tt	−	t	t	−	−	t	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Positive control Serum 396 *vs.* horse dander extract	Ring Flocculation Sedimentation	4+	4+	4+	4+	4+	4+	3+	3+	3+	2+	2+	2+	−	tt	+	−	t	t	−	−	t	−	−	−			
Cross-titration Serum 396 *vs.* normal horse serum	Ring Flocculation Sedimentation	2+	t	tt	3+	tt	+	3+	+	+	2+	2+	2+	2+	+	+	+	+	+	−	t	t	−	−	t			

TABLE II.

Tiration Record of an Antiserum against Horse Serum.

Serum 55 (against horse serum).

t = turbidity + = flocculation, sedimentation, or positive ring test − = negative of two preceding		1/5			1/20			1/100			1/500			1/2,000			1/10,000			1/50,000			1/200,000			Anti-serum Saline control		
Negative control Serum 55 *vs.* normal guinea pig serum	Ring	−			−			−			−			−			−			−			−			−		
	Flocculation		t			−			−			−			−			−			−			−			−	
	Sedimentation			t			t			−			−			−			−			−			−			−
Positive control Serum 55 *vs.* normal horse serum	Ring	3+			3+			3+			2+			+			−			−			−					
	Flocculation		3+			3+			3+			2+			+			t			−			−				
	Sedimentation			3+			3+			3+			2+			+			+			t			−			
Cross-titration Serum 55 *vs.* horse dander extract	Ring	2+			+			−			−			−			−			−			−					
	Flocculation		2+			+			t			−			−			−			−			−				
	Sedimentation			2+			+			+			±			±			−			−			−			

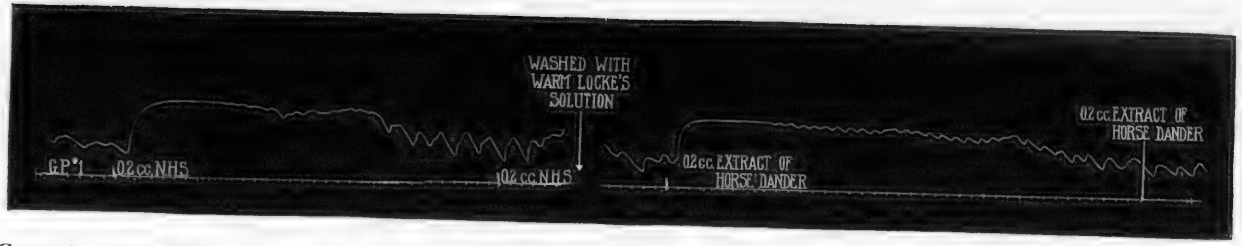

CHART 1. Guinea Pig 1. Sensitizing dose, May 18, 1.0 cc. horse dander extract (Berkefeld-filtered). Date of test, June 5. N. H. S., normal horse serum.

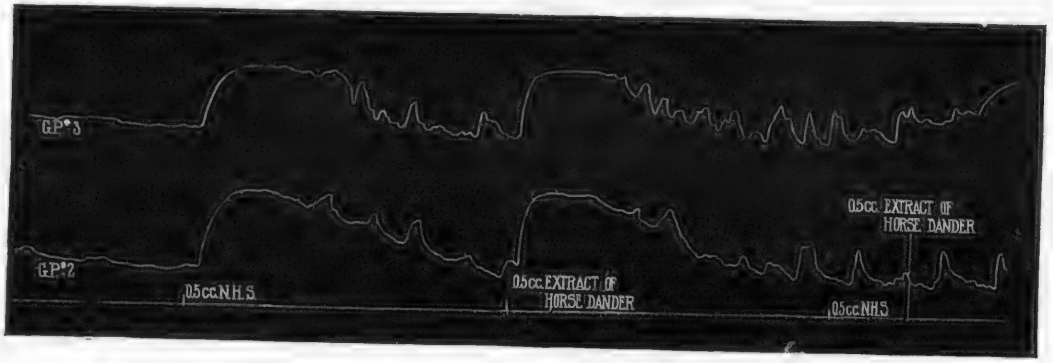

CHART 2. Guinea Pigs 2 and 3.

Guinea Pig 2. Sensitizing doses, May 17, 2.0 cc. Precipitating Serum 396 (against horse dander), intraperitoneally; May 18, 1.5 cc. same serum, intraperitoneally. Date of test, May 20. N. H. S., normal horse serum.

Guinea Pig 3. Sensitizing dose, May 18, 2.0 cc. Serum 396 intraperitoneally. Date of test, May 20.

different and this difference is characteristic. Low cross-titers were the rule in cross-precipitation of horse dander extract by antisera against horse serum.

Uterine Strip Reactions.—Both actively and passively sensitized guinea pigs were used.

Active Sensitizations.—Of the seven pigs actively sensitized to dander extract (Berkefeld-filtered) only two reacted to horse serum when tested by the uterine strip method, but these are sufficient to show that such a cross-reaction is obtainable. The kymographic record of one of these pigs (No. 1) is shown in Chart 1. This animal was sensitized by 1.0 cc. of dander extract (Berkefeld-filtered), given intraperitoneally, and was used in the test recorded 18 days later. It is noteworthy that although a reaction was obtained with horse serum, the uterus was only partially desensitized, as witnessed by the subsequent reaction to horse dander extract. This is probably explainable by the fact that horse dander contains as its chief antigenic constituent a protein which is not present in horse serum, namely the "alkali meta-protein" of Wodehouse, or protein B of Longcope *et al.* This partial desensitization of the uterus by horse serum was a regular occurrence in this type of cross-reaction (that is, including guinea pigs passively sensitized to horse dander—see below).

No pig of the eight actively sensitized to horse serum gave a response to dander extract by the Dale method.

Passive Sensitizations.—More success attended the efforts at passive sensitization. Cross-reactions by the uterine strip method of Dale were obtained in six guinea pigs passively sensitized by antidander sera from rabbits. One failure resulted, probably due to an insufficient incubation period. Two other attempts with the same antiserum that was used in this negative case were successful. Chart 2 shows the response of two of these animals (Nos. 2 and 3) to horse serum.

Passive sensitization with sera from rabbits immunized against horse serum was not attempted.

Cross-Anaphylactic Tests in Living Animals.—The results obtained in these tests are in substantial agreement with those already described.

Reactions in Guinea Pigs Sensitized to Horse Dander.—Sensitization was accomplished by a dosage of 1.0 to 2.0 cc. of centrifugalized dander suspension or of Seitz-filtered dander extract, intraperitoneally or

intracardially administered. Shock doses of 1.5 to 2.0 cc. normal horse serum were given intracardially 12 to 14 days later. Of twelve pigs thus treated ten experienced fatal shock with typical dyspnea, autopsy showing characteristic marked distention of the lungs. One

TABLE III.

Cross-Anaphylatic Tests in Guinea Pigs Sensitized to Horse Dander.

Guinea pig No.	Sensitizing injection	Shock dose	Symptoms
4	1.0 cc. centrifugalized horse dander suspension, I. P.	2.0 cc. normal horse serum, I. C.	Nose scratching, dyspnea. Death* within 3 min.
5	*Ibid.*	1.5 cc. *Ibid.*	Urination, dyspnea. Death* within 3½ min.
6	*Ibid.*	*Ibid.*	Slight dyspnea lasting a few min. Survival
7	*Ibid.*	*Ibid.*	Dyspnea. Death* within 4 min.
8	*Ibid.*	*Ibid.*	Doubtful symptoms—slight dyspnea. Survival
9	*Ibid.*	*Ibid.*	Dyspnea. Death* within 5 min.
10	*Ibid.*	*Ibid.*	Dyspnea. Death* within 3 min.
11	*Ibid.*	*Ibid.*	Dyspnea. Death* within 3½ min.
12	*Ibid.*	*Ibid.*	Dyspnea. Death* within 4 min.
13	2.0 cc. Seitz-filtered dander extract, I. C.	2.0 cc. *Ibid.*	Dyspnea. Death* within 6 min.
14	*Ibid.*	*Ibid.*	Dyspnea. Death* within 3¾ min.
15	*Ibid.*	*Ibid.*	Dyspnea. Death* within 3 min.
16	No treatment	1.5 cc. *Ibid.*	Control. No symptoms
17	*Ibid.*	*Ibid.*	Control. No symptoms

* Autopsy showed typical marked distention of lungs except where contrary is indicated.

I. P. means intraperitoneal injection; I. C., intracardial.

of the two other pigs showed slight respiratory symptoms, but recovered, while the remaining one showed no definite symptoms. Two controls, receiving only the intracardial dose of horse serum showed no symptoms whatever. These results are given in more detail in Table III.

Reactions in Guinea Pigs Sensitized to Horse Serum.—Fifteen guinea

TABLE IV.

Cross-Anaphylactic Tests in Guinea Pigs Sensitized to Normal Horse Serum.

Cross-anaphylactic tests				Tests to determine whether desensitization had been accomplished. (On day following shock dose.)	
Guinea pig No.	Sensitizing injection	Shock dose	Symptoms	Dose	Symptoms
18	1.0 cc. normal horse serum, I. P.	2.0 cc. horse dander extract, I. C.	Nose scratching, slight dyspnea. Survival		
19	*Ibid.*	*Ibid.*	Tremors, slight dyspnea. Survival		
20	*Ibid.*	*Ibid.*	Slight dyspnea. Survival		
21	*Ibid.*	*Ibid.*†	No symptoms		
22	*Ibid.*	*Ibid.*	Slight dyspnea. Survival		
23	*Ibid.*	*Ibid.*	Sneezing, nose scratching, dyspnea lasting several min. Survival		
24	*Ibid.*	*Ibid.*	Slight dyspnea, nose scratching. Survival		
25	*Ibid.*	*Ibid.*	No symptoms		
26	*Ibid.*	*Ibid.*	No symptoms	2.0 cc., normal horse serum, I. C.	Dyspnea. Death* within 3½ min.
27	*Ibid.*	*Ibid.*	Nose scratching, delayed dyspnea (12 min.). Death in 15 min. Lungs not distended at autopsy		
28	*Ibid.*	4.0 cc. *Ibid.*	Prostration, respiratory difficulty. Survival	4.0 cc. *Ibid.*	Typical dyspnea, lasting ½ hr. Survival
29	*Ibid.*	2.0 cc. *Ibid.*	Severe dyspnea for 11 min. Survival	2.0 cc. *Ibid.*	Moderate respiratory symptoms. Death after 13 min. Lungs not distended. Early pregnancy

30	*Ibid.*	4.0 cc. *Ibid.*	No symptoms	4.0 cc. *Ibid.*	Typical dyspnea. Death* in 4½ min.
31	*Ibid.*	2.0 cc. *Ibid.*	Nose scratching, prostration. Survival	2.0 cc. *Ibid.*	Typical dyspnea. Death* in 4½ min.
32	*Ibid.*	*Ibid.*	Weakness, collapse, no respiratory symptoms, recovery. Death overnight. No autopsy		
33	No treatment	*Ibid.*	Control. No symptoms		
34	No treatment	*Ibid.*	Control. Death in 11 min. Lungs not distended‡		
35	No treatment	*Ibid.*	Control. No symptoms		
36	No treatment	*Ibid.*	Control. No symptoms		

* Autopsy showed typical marked distention of lungs except where contrary is indicated.
† Some doubt about intracardial nature of injection.
‡ Death thought to be due to cardiac hemorrhage.
I.P. means intraperitoneal injection; I.C., intracardial.

pigs received sensitizing doses of 1.0 cc. normal horse serum intraperitoneally (see Table IV). Shock doses of 2.0 to 4.0 cc. of Seitz-filtered horse dander extract were administered 12 to 15 days later. In none of these did typical, fatal anaphylaxis occur. Three (Nos. 28, 29, 31) showed characteristic dyspnea and other symptoms and six (Nos. 18, 19, 20, 22, 23, 24) reacted more doubtfully. Six (Nos. 21, 25, 26, 27, 30, 32) failed to show any reaction which could be called anaphylactic. Four control animals were used, these receiving no sensitizing dose, but an intracardial injection of dander extract. One of these (No. 34) died a few minutes after injection, but with none of the characteristic symptoms. Autopsy showed the lungs collapsed and the pericardial cavity tightly distended with blood. It is believed that cardiac hemorrhage was responsible for death.

Five of the guinea pigs sensitized to horse serum were further used to learn whether desensitization had been brought about by injection of the horse dander extract. 24 hours after the latter injection, 2.0 to 4.0 cc. of horse serum was given intracardially to each of these (Nos. 26, 28, 29, 30, 31). For results see Table IV. Three of them (Nos. 26, 30, 31) died in typical anaphylaxis following this treatment. It is obvious that no complete desensitization resulted from any of the "shock doses" (dander extract). Partial desensitization seems to have occurred in some of the five. Thus Nos. 28 and 29 afforded the most characteristic picture of non-fatal anaphylaxis when horse dander extract was given as a "shock dose." No. 28 survived the subsequent injection of horse serum 24 hours later. No. 29 succumbed to the latter treatment, but the death was not anaphylactic, since the lungs were collapsed. Nos. 26 and 30 showed no symptoms following the "shock dose" of horse dander extract, while horse serum on the following day produced dyspnea and death with lungs distended.

DISCUSSION.

It seems evident from the foregoing results that there is an antigenic element common to horse dander and horse serum. This common antigenic substance yet remains to be isolated biochemically. That it is present in relatively small proportion in horse dander is suggested (1) by the low titer obtained in cross-precipitation tests involving an antiserum against horse serum and extract of horse dander, as well as

(2) by the failure of horse dander extract to produce fatal shock or even to desensitize completely guinea pigs which had been sensitized to horse serum. The results here obtained shed no light on the relative concentration of the common antigen in horse serum, since only a minute amount of it would probably be necessary to sensitize a guinea pig and no great amount of it would be necessary for precipitin production in a rabbit.

The work of Longcope, O'Brien, and Perlzweig (6) indicates that horse dander contains no protein precipitable at the isoelectric points of either serum globulin or serum albumin. This seems to be at variance with the results obtained in the present study since dander, if it has any antigenic element in common with serum, must contain either globulin or albumin.

It is possible that serum may be present in dander as a contaminant, as a result of exudation from cuts or sores, or from slight bleeding during the rather strenuous process of currying. If serum proteins were derived from such a source, different lots of dander would, of course, contain different proportions of them. In this study three lots of dander have been used, harmonious results being obtained from all of them. Two lots came from the horses of the Boston Fire Department and one lot from the horses of Parke, Davis and Company.

Whatever the common antigen may prove to be chemically, however, the fact of its existence seems to be attested by the results detailed herewith. Furthermore, the fact of sensitivity both to horse serum and to horse dander extract, such as is shown in a considerable percentage of horse-asthmatics is logically explained thereby.

SUMMARY.

Evidence has been submitted of the existence of a common antigenic substance in horse dander and horse serum. This evidence has been derived from three lines of study:

(*a*) Cross-precipitation tests involving (1) the titration of antisera against horse serum with saline extract of horse dander and (2) titration of antisera against horse dander with normal horse serum.

(*b*) Cross-anaphylactic tests by the uterine strip method of Dale.

(*c*) Cross-anaphylactic tests in living guinea pigs by the usual shock method.

It seems likely from the work here described that the common antigen is present in small proportion in horse dander. Its concentration in horse serum is not indicated by the results obtained.

The writer desires to express his appreciation to Dr. Hans Zinsser for his interest and helpful criticisms during the course of the work.

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STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI.

I. A Virulent Strain of Pneumococcus Which Is Immunologically Related to, but Not Identical with Typical Strains of Type III Pneumococci.

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INTRODUCTION.

The present paper deals with a strain of Pneumococcus which is related to, but not identical with typical strains of the Type III group. The general interest in strains of bacteria that possess a specific immunological relationship to one of the "fixed" types (1) of any important pathogenic group acquires special interest in the case of pneumococci in view of the fact that the specificity of Pneumococcus Types I, II and III furnishes the most clear-cut example in all bacteriology of the dependence of exquisite biological specificity upon the chemical constitution of the bacteria themselves (2).

In order to avoid any confusion that might arise from either the term "subgroup" or the term "atypical," the terms "Thomas strain" and "anti-Thomas serum" are used throughout the present paper to designate the "non-typical" strain and its antiserum.

It is important to point out at the beginning, that the Thomas strain is virulent, killing mice within 36 hours in doses of 1×10^{-8} cc. of plain broth culture. As shown by Tillett (3), the type-specific agglutination of the "S" forms of Type III pneumococci involves an anti-S antibody in contrast to the anti-P antibody

* Mr. Gaspari's cooperation in this work was made possible by a grant from The Henry Strong Denison Medical Foundation.

which agglutinates the "R" or degraded forms of all types of pneumococci. The characteristics which distinguish the Thomas strain from the typical Type III pneumococcus are recognized by S-anti-S reactions, and have nothing to do with those which distinguish the "S" forms (S-producing) of pneumococci from the avirulent, degraded or "R" forms (non-S-producing). Although not described in detail, the controls included in all experiments were sufficient to eliminate any complications arising from failure to differentiate the anti-S reactions from anti-P reactions. The high degree of virulence of the strain, the characteristic anti-S character of the agglutination of the bacteria and of the precipitation of young broth culture filtrates by the homologous antiserum, as well as actual tests of the non-type-specific protein-precipitating (anti-P) capacity of the antiserum, are in themselves convincing evidence that the Thomas strain is not a "degraded" form of typical Type III pneumococcus.

The Thomas strain exhibits no immunological relationship to Type I or II pneumococci with the exception of the species-specific P-anti-P relationship which is common to all pneumococci: the Thomas pneumococci are not agglutinated by Type I or II antiserum nor do these antisera confer passive protection; anti-Thomas immune serum is likewise non-reactive against Types I and II pneumococci; similarly, absorption of Type I or II antiserum with Thomas bacteria and absorption of anti-Thomas serum with Type I or II bacteria have no effect upon the type-specific antibodies.

EXPERIMENTAL

Methods.

In this investigation, five different methods have been used in testing the immune sera for the presence of the antibody specifically reactive with Type III pneumococci. These methods can be arranged in the following order of delicacy: (1) specific, passive protection of mice against virulent Type III bacteria; (2) agglutination of concentrated suspensions of heated Type III bacterial cells in salt solution; (3) agglutination of heated broth culture; (4) agglutination of unheated broth culture; (5) precipitation of solutions of the Type III specific S substance. The different procedures simply represent different methods of detecting the same anti-S antibody, and the positive results obtained when an immune serum is tested by one method, and the negative results obtained when the same serum is tested by another method, are due to differences in delicacy of the method of test.

The Relationship of the Thomas Strain to Typical Type III Strain of Pneumococci as Evidenced in Tests with Potent Anti-Type III Serum and Potent Anti-Thomas Serum.

The comparison in Table I of the immunological properties of the Thomas pneumococci with those of typical Type III pneumococci, is

TABLE I.

The Relationship of the Thomas Strain to Typical Type III Pneumococci as Evidenced in Tests with Potent Anti-Type III Horse Serum and Potent Anti-Thomas Rabbit Serum.

Dilution of serum	1. Agglutination of broth culture				2. Precipitation of S substance contained in filtrate of young broth culture				3. Type-specific passive protection				
	Typical Type III strain		Thomas strain		Typical Type III strain		Thomas strain		Dose of culture	Typical Type III strain		Thomas strain	
	Type III serum	Anti-Thomas serum	Type III serum	Anti-Thomas serum	Type III serum	Anti-Thomas serum	Type III serum	Anti-Thomas serum		Type III serum	Anti-Thomas serum	Type III serum	Anti-Thomas serum
									cc.				
Undiluted	+++	+++	+++	+++	+++	+++	+++	+++	0.01	D	D	D	S
1/5	+++	++	+++	+++	+++	+	±	+++	0.001	D-S	D-S	D-S	S
1/20	++	0	0	+++	+++	0	0	+++	0.0001	S	S	S	S
1/40	±	0	0	+++									
1/320	0	0	0	++									
1/640	0	0	0	±									

0 = indistinguishable from controls; ± = slight granulation; + = granular sediment with definite particles when thoroughly shaken; ++ = bacteria sedimented in disc form but supernatant not entirely clear and disc easily broken when shaken; +++ = compact disc with supernatant entirely cleared.

D = mice died within 24 to 72 hours in all tests; D–S = protection irregular, mice surviving in some experiments and dying in others; S = mice survived in all tests. Virulence controls (without serum or with heterologous serum) dying within 72 hours when injected with 1×10^{-8} cc. of culture.

based upon tests made with the most potent anti-Thomas and the most potent anti-Type III serum which we have obtained. While there are wide variations in the individual potencies of the anti-Thomas and anti-Type III serum obtained from different animals, it is desirable to base the first comparison upon the immunological properties exhibited in immune sera containing the complete expression of the antigenic properties of the two related but different kinds of pneumococci.

As shown in Table I, the Thomas strain of pneumococci reacts with anti-Type III serum, although its agglutination, filtrate precipitation and passive protection are not as pronounced as are the corresponding reactions of typical Type III pneumococci in anti-Type III serum. Thus, in respect to the reactions in potent samples of Type III immune serum the Thomas strain appears to be related to typical Type III pneumococci in about the same way as Avery's (1) Subgroup *IIa* strains are related to typical Type II pneumococci.

However, the comparison of the two strains in the anti-Thomas serum gives a more clear-cut differentiation between the Thomas and the typical Type III strains. In the first place, the anti-Thomas serum reacts not only with the Thomas pneumococci but also with typical strains of Type III pneumococci. In the second place, the potency of the anti-Thomas serum against the Thomas bacteria is much greater than the potency of anti-Type III serum against Type III pneumococci themselves; the invariable protection of mice against 0.01 cc. of broth culture (1 million lethal doses) and the definite agglutination of broth cultures by 1/320 dilution of serum being of a much higher order of potency than that obtained with typical Type III pneumococci in anti-Type III serum whether from rabbits or hyperimmunized horses. This high degree of homologous potency of the antiserum produced by immunization with the atypical strain was a frequent occurrence in Avery's (1) work with the different Subgroup Type II pneumococci, but the reactivity of typical Type III strains in the anti-Thomas serum is in direct contrast to the lack of any reactivity of typical Type II strains in any of the different Subgroup Type II antisera.

Variations in the Relative Potencies of Different Type III Immune Sera in Respect to Antibodies Reactive with a Typical Type III Strain and Antibodies Reactive with the Thomas Strain.

The comparison in Table I of the Thomas strain with a typical Type III strain was based upon their reactions in immune sera chosen for their potency against both the Thomas strain and typical Type III strains. During the investigation, many tests have been made with a number of different Type III immune sera. The results of these tests showed a wide range of differences in the relation between the anti-Type III and the anti-Thomas potency in different anti-Type III sera. The reactivity of the serum against typical Type III pneumococci had no regular relation to its reactivity against the Thomas pneumococci. For example, one of the anti-Type III sera that was most reactive against typical Type III pneumococci did not agglutinate the Thomas strain at all after 2 hours incubation at 37°C. and only occasionally agglutinated it after storage in the ice box overnight. Similarly, one of the anti-Type III sera that was weakly reactive against typical Type III strains was as reactive against the Thomas strain as any of the sera that we tested. This lack of any relation between the anti-Thomas potency and the anti-Type III potency of the individual sera is important if it represents a difference in the relative proportion of two slightly different antibodies in the serum obtained from different horses after immunization with Type III pneumococci.

Variations in the Relative Potencies of Anti-Thomas Serum from Different Rabbits in Respect to Antibodies Reactive with Typical Type III Strains and Antibodies Reactive with the Thomas Strain.

During the investigation, ten different rabbits were immunized with heat-killed suspensions of the Thomas pneumococci. A summary of the results of the tests of the potencies of these anti-Thomas rabbit sera is presented in Table II.

An analysis of Table II reveals two important facts: (1) the anti-Thomas sera obtained from ten rabbits showed no great difference in their respective potencies against the homologous strain; (2) in spite

of the uniformly good anti-Thomas immunity response, the sera of the same rabbits showed great differences in their respective potencies against the typical Type III strain.

If the homologous potency be dismissed as uniformly good in all of the anti-Thomas sera, it is possible to arrange the sera from the ten rabbits into three groups in respect to [illegible] potency against typical

TABLE [illegible].

Variations in Relative Potencies of Anti-Thomas Immune Serum from Different Rabbits in Respect to Antibodies Reactive with Typical Type III Pneumococci and Antibodies Reactive with the Thomas (Homologous) Strain.

Anti-Thomas sera	Antibodies reactive with typical Type III pneumococci					Antibodies reactive with Thomas (homologous) strain		
	Agglutination			Precipitation of S substance contained in culture filtrate	Passive protection of mice against 1×10^{-4} or 1×10^{-5} cc. of culture	Agglutination of unheated culture by 1/160 or 1/320 dilution of serum	Precipitation of S substance contained in culture filtrate in high dilution of serum or of antigen	Passive protection of mice against at least 1×10^{-3} cc. of culture
	Unheated culture	Heated culture	Concentrated suspension of heated bacterial cells					
Serum from 2 rabbits	+	+	+	+	+	+	+	+
Serum from 7 rabbits	0	±	±	0	+	+	+	+
Serum from 1 rabbit	0	0	0	0	0	+	+	+

0 = negative results in all tests with all sera; ± = definitely positive results with 5 of the sera, but equivocal results with 2 sera of this group; + = definitely positive results in all tests with all sera.

Type III pneumococci. The first group includes the sera from two rabbits: these anti-Thomas sera agglutinated unheated broth cultures of Type III pneumococci almost as well as the best Type III immune horse sera and were much more reactive than some of the diagnostic sera supplied by biological houses for routine typing. The second group includes the sera from seven rabbits: they failed to agglutinate unheated cultures of Type III strains, some of them agglu-

tinated heated cultures, most of them agglutinated concentrated suspensions of the bacterial cells and all of them passively protected mice against typical Type III pneumococci. The third group includes the serum from one rabbit which, although highly potent against the Thomas strain, failed entirely to show any type-specific immunity against typical Type III strains; the lack of protection against minimal doses, which is the most delicate criterion, being accepted as evidence of the complete lack of anti-Type III antibodies. From the standpoint of the probability of the presence of two different anti-S antibodies in the same antiserum, it is important to note that these marked variations in anti-Type III potency occurred in anti-Thomas sera which showed no significant differences in their potency against Thomas pneumococci.

It seems unlikely that these differences in the amounts of anti-Type III antibody in anti-Thomas sera containing uniformly large amounts of the anti-Thomas antibody, are due to differences in the antigen injected. All of our ten rabbits were not immunized at the same time, and it happened that the first four rabbits (injected in June and July) gave better anti-Type III responses than two rabbits immunized later (injected in September and October). We thought at that time that the poorer response of the latter rabbits might have been due to a change in the antigenic properties of the Thomas strain. In order to rule out the possibility of the repeated mouse passage of the culture having changed the Thomas strain in the direction of loss of its Type III antigenic capacity, two rabbits were later (November and December) immunized with the mouse passage strain and two other rabbits with vaccine prepared from a culture which had been in the ice box for 3 months without animal passage. The sera of all four animals gave the usual strong anti-Thomas response; and the variations in the anti-Type III response were unrelated to the mouse passage of the culture.

Tests for the Presence of Type-Specific Anti-Type III Antibody in Anti-Thomas Sera by Precipitation of Solutions of the Purified Carbohydrate S Substance Derived from Typical Type III Pneumococci.

In preceding experiments, filtrates of young broth cultures of the Thomas strain and of typical Type III strains were employed as sources of the specific S substance elaborated by virulent type-specific pneumococci. It seemed important, however, to test the Thomas antisera against solutions of the purified carbohydrate S substance derived from typical Type III strains (4). A sample of the carbohydrate S substance furnished for this purpose by Dr. O. T. Avery

of the Hospital of The Rockefeller Institute, was tested against the anti-Thomas immune sera. The tests were made by adding 0.2 cc. of serum to 0.5 cc. of three different dilutions of the S substance (1/10,000, 1/50,000 and 1/100,000). The immune sera from four of the animals immunized earlier in the investigation were no longer available at the time the purified solution was obtained.

The results of the tests with the purified carbohydrate confirmed the results of the preceding tests with the filtrates of the Type III cultures, for the sera of some of the rabbits immunized with Thomas pneumococci precipitated the solutions of the highly specific and chemically purified carbohydrate substance prepared from typical Type III pneumococci. The prozone phenomenon was much more marked than in tests with anti-Type III horse serum. The two most reactive anti-Thomas sera precipitated the 1/50,000 solution better than the 1/10,000 solution, and gave no definite reaction at all in tests with 1/1,000 solution. This marked prozone made it seem inadvisable to attempt to increase the number of positively reacting anti-Thomas sera by repeating the tests with higher concentrations of antigen.

Absorption of Type III Immune Horse Sera with Suspensions of Typical Type III Pneumococci and with Suspensions of the Thomas Strain.

Anti-Type III serum was absorbed with suspensions of typical Type III pneumococci and with suspensions of Thomas pneumococci. In view of the factors that may influence the results of absorption tests, ten experiments were made with three different anti-Type III immune sera, under quantitatively different sets of conditions.

The results of these experiments were the same as those usually obtained in reciprocal absorption experiments with immunologically related, but different, kinds of bacteria. Absorption of the anti-Type III serum with the typical strain (homologous) completely exhausted it not only of antibodies reactive with typical Type III strains but also of those reactive with the Thomas strain. On the other hand, repeated absorption with the Thomas bacteria (heterologous) removed only the antibodies reactive with the Thomas strain and had little, if any, effect upon the potency of the serum when tested against the typical strain. The failure of repeated absorption with the Thomas bacteria to reduce the anti-Type III potency seems to us

to indicate the presence of at least two different type-specific (anti-S) antibodies in Type III immune horse serum, only one of which can be removed by the Thomas strain.

Absorption of Anti-Thomas Immune Sera with Suspensions of Typical Type III Pneumococci and with Suspensions of the Thomas Strain.

Anti-Thomas serum, potent against both typical Type III strains and the homologous Thomas strain, was absorbed with suspensions of the typical Type III bacteria and with suspensions of the Thomas bacteria under conditions analogous to those employed in the absorption of anti-Type III serum. The results of these experiments, which have been repeated many times with four different anti-Thomas immune sera, are summarized as follows:

Absorption of anti-Thomas serum with the homologous strain stripped the serum of antibodies reactive with typical Type III organisms as well as those reactive with the homologous (Thomas) organisms. Absorption with the typical Type III strain, on the other hand, removed only the antibodies reactive with typical Type III organisms and did not significantly diminish the anti-Thomas potency of the serum.

Tests with Other Typical Strains of Type III Pneumococci.

In most of the previously described experiments, one strain (A 66, Hospital of The Rockefeller Institute) was utilized as the representative of the typical Type III group. In order to determine if the relations found between the Thomas strain and the representative typical strain would hold true for other "typical" strains of Type III pneumococci we have repeated most of the described experiments with three different Type III strains recently isolated from different patients at the Vanderbilt University Hospital.

The anti-Thomas serum agglutinated and protected against the recently isolated strains as well as in the previous tests with the Rockefeller laboratory strain. Similarly, the Type III immune horse sera were completely stripped of antibodies (both anti-Type III and anti-Thomas) by absorption with the Nashville strains; and absorption of the anti-Thomas sera removed the anti-Type III and not the anti-

Thomas antibodies just as had absorption with the previously used Rockefeller strain. The repetition of the preceding experiments with these different and recently isolated strains adds considerable strength to all of the results and makes the absorption experiments much more convincing.

While the three strains (isolated in Nashville) represent too small a number to argue for the immunological homogeneity of the Type III group, the experiments with the recently isolated strains do serve to rule out the possibility that the preceding absorption results were due simply to our having used the same strain as that commonly used in the production of the Type III diagnostic serum in the different laboratories, and show that the anti-Thomas immune serum contains antibodies reactive with more than one strain of "typical" Type III pneumococci.

The necessity of repeating the preceding work with those additional strains which were known not to be the same as those utilized in the production of the anti-Type III serum is especially evident in view of the possibility that all of the biological producing laboratories may use the same strain in the production of anti-Type III diagnostic serum. And, it seemed probable to us that perhaps this one strain, the original source having been the Hospital of The Rockefeller Institute, might be the same one as that which we have employed as the representative Type III strain in our preceding experiments.

The Protection Test as a Criterion of the "Type Purity" of Pneumococcus Cultures.

The Thomas strain has been plated out repeatedly and we are convinced that its serological relationship to Type III is real, and not an apparent relationship due to the use of a mixed culture containing a few Type III organisms together with an unrelated Group IV strain. The fact that Type III immune horse sera produced in four different laboratories (by immunization with presumably "pure" Type III organisms) possessed marked protective power against the Thomas strain, seems to us to be in itself convincing evidence of the "type purity" of the culture.

As pointed out by Avery (1) specific protection is the ultimate criterion of type specificity among the pneumococci. Since protection tests offer the most delicate index of the presence of pneumococcus type-specific antibodies and suffice to detect them in antisera when test-tube methods fail, they should likewise prove to be the most delicate criteria of the "type purity" of pneumococcus cultures. For example, agglutination with the usual type sera would probably fail to detect

the presence of virulent Group IV pneumococci if mixed in small amounts with a Type III culture, but if passive protection tests were made, the mice would be infected by the small numbers of virulent Group IV organisms in spite of the protection conferred against the Type III bacteria themselves.

While it seemed certain that this principle would always hold true with virulent cultures, we have made two different sorts of experiments in order to test it. First, experiments were made in which the protective power of Type III serum was tested against: (*a*) Type III bacteria by themselves; (*b*) mixed cultures containing Type III and Type II bacteria in proportion of 1,000 to 1; (*c*) mixed cultures containing Type III and Type II in proportion of 10,000 to 1. The results of these experiments were exactly what one would expect if the presence of sensitized Type III pneumococci did not affect the virulence of the heterologous organism. The Type III serum protected against Type III alone, and failed against mixtures of Type III bacteria with 0.1 and 0.01 per cent of the heterologous organisms. All of the mice were autopsied. It is obvious that the experiment was concerned only with the animals which were killed by the mixed culture and not by the same amount of "pure" Type III; and typing of the heart's blood culture from these mice indicated that the heterologous organism was the sole cause of death and that the sensitized Type III bacteria had failed to survive in the blood stream even in mice having a septicemia due to the heterologous type.

Second, experiments were made to test the effect of the presence of small numbers of the Thomas bacteria upon the protective action of Type II serum; tests being made against: (*a*) Type II bacteria alone; (*b*) mixtures of Type II and Thomas bacteria in proportion of 10,000 to 1; (*c*) mixtures of Type II and Thomas bacteria in proportion of 100,000 to 1. The results of these experiments were analogous to the first ones; and in this case (due to the higher degree of homologous protection of the Type II serum), the protection test served to detect the presence of Thomas (heterologous) bacteria in the Type II culture even when present in the proportion of 1 to 100,000 of the homologous organisms.

DISCUSSION.

The preceding experiments dealt with the immunological properties of the Thomas strain of Pneumococcus which is related to but not identical with typical Type III strains. In respect to the reactions in potent anti-Type III immune horse serum, the relationship between the Thomas strain and typical Type III strains is about the same as that evidenced in anti-Type II serum between typical Type II strains and most of Avery's Subgroup II strains. But, when the comparison is made in anti-Thomas immune serum, it is evident that the relationship of the Thomas strain to typical Type III pneumococcus is different, for the anti-Thomas immune serum (from most rabbits) agglutinates and

protects against typical Type III, while none of the anti-Subgroup II immune sera were reactive against typical Type II pneumococci. The production of sera reactive against typical Type III pneumococci by injection of the Thomas strain is particularly interesting in view of the rarity of obtaining an effective anti-Type III immunity response in rabbits by the injection of typical Type III pneumococci. From these results it appears that antibodies reactive with typical Type III pneumococci can be produced more readily when rabbits are immunized with the Thomas bacteria than when immunized with typical Type III bacteria themselves. Whether the antigen in the Thomas bacterial cell which is responsible for the antibody reactive with the Type III bacteria is the same as the corresponding antigen in the Type III bacterial cell is another question.

The anti-Type III immune serum from different horses and the anti-Thomas serum from different rabbits, usually contained antibodies reactive both with the Thomas bacteria and with the typical Type III bacteria. It is particularly important that there was an entire lack of any regular relation between the relative anti-Thomas and anti-Type III potencies of the different individual antisera. The pronounced variations in the relative potencies of individual antisera in respect to antibodies reactive with the two kinds of pneumococci may represent differences in the relative proportion of two different anti-S antibodies in the antiserum from different individual animals. The variation in the anti-Type III potency of the different anti-Thomas sera is probably due to differences in the response of the individual rabbits to the particular Thomas antigen which gives rise to the anti-Type III antibody, for Tillett (3) found marked differences in the individual anti-Type III responses of rabbits when Type III bacteria themselves were injected. The possible variations in the antigenic character of the cultures (both of the Thomas and of the typical Type III strains) does not seem to be a likely explanation of the variations in the immune sera for we frequently obtained wide differences in the relative anti-Type III potency in the anti-Thomas sera of different rabbits immunized at the same time with equal amounts of the same Thomas vaccine.

The results of absorption experiments with both typical Type III antiserum and Thomas antiserum were the same as those usually ob-

tained in similar tests with immunologically related, but not identical bacteria. That is, homologous absorption removed all of the antibodies from each serum, and heterologous absorption removed only the antibodies reactive with the strain used in absorption and failed to exhaust the serum of antibodies reactive with the strain used in immunization. The failure of reciprocal absorption together with the variations in the relative potencies $\left(\text{ratio of } \frac{\text{Anti-Thomas potency}}{\text{Anti-Type III potency}}\right)$ of the antiserum from different individual animals could be presented as presumptive evidence that two different anti-S antibodies are contained in Type III immune horse serum. Although there is no evidence of complexity among the type-specific antigens of the "fixed" types of pneumococci, the same S substance united with slightly different protein constituents might give rise to related but slightly different type-specific antibodies.

In his original paper on the Subgroup Type II pneumococci, Avery (1) pointed out that the serological relationship did not in itself indicate that the Subgroup Type II and typical Type II strains were related by the lineage of common descent. The later developments in knowledge (2) of the antigenic constituents of the pneumococcus cell show more clearly that a serological relationship like that between the Thomas strain and typical Type III strains is not always a true index of phylogenetic relationship. It is now well known that there are two sorts of antigen-antibody systems involved in the immunological reactions of pneumococci: the S-anti-S reactions of type specificity, and the P-anti-P reactions of species specificity. There is much evidence that the second of these reactions is the more likely to indicate phylogenetic or truly biological relationship. Since the S-anti-S reactions separate into distinct "types" the pneumococci which manifest group relationship by P-anti-P reactions, it is important to recognize the possibility of biologically fortuitous likenesses in the chemical structure of some one of the cell constituents of phylogenetically unrelated bacteria. This possibility is well illustrated by the similarity between the S substance of Type II pneumococci and the S substance of some strains of Friedländer's bacillus (5). The common sense of the biologist would preclude the assumption of a closer phylogenetic relationship between Type II pneumococcus and Friedländer's bacil-

lus than between Type II pneumococcus and Type I or III, simply because of a greater chemical likeness between the carbohydrates elaborated by the bacteria. But, in point of fact, as far as the S-anti-S reactions are concerned, the serological relationship of the Thomas bacteria to the typical Type III bacteria is no more pronounced than that between Friedländer bacilli and Type II pneumococci; and hence, there is no real reason to believe that the Thomas strain is any more closely related, in a truly biological sense, to Type III than to any other virulent Pneumococcus.

In the absence of any evidence of phylogenetic relationship, the Thomas strain can best be considered as a Pneumococcus which, in addition to distinct immunological properties of its own, possesses a partial antigenic relationship to the typical Type III pneumococcus. The degree of type specificity manifested by Types I, II and III pneumococci is of a higher order than that usually obtained between the "types" contained in most groups of bacteria. But, in view of the wide range of immunological possibilities that are presented by Group IV pneumococci, one can expect to find a certain number of pneumococci that are related to but not identical with one of the "fixed" types. While there have been few, if any reports of pneumococci related to Type III it is quite possible that the use of a more highly reactive Type III diagnostic antiserum would result in the detection of strains related to Type III which would be included within Group IV on the basis of typing tests with weak Type III antiserum. This possibility is mentioned because of our own experience with the Thomas strain. When first typed in our laboratory, it was considered a Group IV strain and its Type III relationship was not recognized until a subsequent typing test was made with a more potent Type III antiserum than that which we had been using for routine typing.

SUMMARY.

The paper reports a study of a virulent, S-producing strain of Pneumococcus which is immunologically related to, but not identical with typical strains of Type III pneumococcus. In a potent anti-Type III serum, the relationship of this strain to typical Type III strains appears to be about the same as the relationship of Avery's Subgroup Type II

strains to typical Type II. But a more pronounced distinction is evident in the antiserum produced by immunization with the strain related to Type III. This antiserum contained antibodies specifically reactive with typical Type III bacteria as well as antibodies reactive with the homologous strain, while anti-Subgroup Type II immune sera are devoid of antibodies reactive with typical Type II pneumococci.

The results of absorption experiments were the same as those usually obtained with immunologically related, but not identical bacteria. The failure of reciprocal absorption and the marked variations in the relative potencies of the antiserum from different individual animals might be presented as presumptive evidence that two different anti-S antibodies are contained in Type III immune horse serum.

The theoretical significance of virulent pneumococci which are related to but not identical with the "fixed" types, is discussed from the standpoint of their importance in the biological classification of the Pneumococcus group.

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STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI.

II. A Comparison of the Antibody Responses of Mice and of Rabbits to Immunization with Typical Type III Pneumococci and to Immunization with a Related Strain.

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INTRODUCTION.

The previous paper (1) dealt with the properties of a mouse-virulent strain (Thomas)[1] of Pneumococcus, which is immunologically related to, but not identical with typical Type III pneumococci. One of its most prominent properties is its antigenic effectiveness in rabbits. In contrast to the rarity of a detectable type-specific (anti-S)[2] response on injection of typical Type III strains, immunization with the Thomas strain invariably yielded high titres of anti-Thomas antibodies and usually also yielded measurable amounts of antibodies specifically[2] reactive with Type III pneumococci. These differences between the

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[1] The terms "Thomas" strain and "anti-Thomas" serum are employed, as in the preceding paper, to refer to the "non-typical" strain and its antiserum.

[2] The distinctions between the anti-S antibody and the anti-P antibody in the serological reactions of Type III pneumococci have been pointed out in detail by Tillett (3). As stated in the preceding paper, the immunity systems involved in the reactions of anti-Thomas serum with typical Type III pneumococci are the same or similar to the S-anti-S systems involved in the type-specific reactions of the "fixed" types with their homologous antisera. The anti-Thomas serum is just as devoid of effect upon Types I and II pneumococci as is anti-Type III serum, and the Thomas bacteria are just as little affected by anti-Type I or anti-Type II serum as are typical Type III pneumococci.

response of rabbits to the Thomas strain and their response to typical Type III strains seem to be important, especially in regard to the anti-Type III response elicited by injection of the Thomas strain.

It has been general experience (2), not only with rabbits but also with larger animals, that potent anti-Type III immune sera are more difficult to obtain than are potent anti-Type I and anti-Type II immune sera. The question arises, therefore, whether the antigen which gives rise to the Type III anti-S antibody is intrinsically a less effective antigen in all animals, or whether this antigen which seems to be so ineffective might not in other animals elicit responses comparable to those invoked by other types of pneumococcus anti-S antigens.

As a step in the accumulation of data on this question, we have compared the antibody response invoked in rabbits with that invoked in mice when the two species of animals are immunized with typical Type III pneumococci and with the related, but different, Thomas strain. The measurements of the immunity response have included antibodies reactive with the Thomas strain as well as those reactive with typical Type III strains, for anti-Type III horse serum and anti-Thomas rabbit serum usually contain antibodies reactive with both kinds of pneumococci (Thomas and Type III).

EXPERIMENTAL.

Comparison of the Antibody Response of Rabbits to Immunization with Typical Type III Pneumococci with their Response to Immunization with the Thomas Strain When the Amounts of Bacterial Antigen Are Equal.

The fact that rabbits respond poorly to Type III pneumococci is clearly shown in Tillett's (3) extensive study in which 18 out of 28 rabbits failed to produce in their serum even the small amount of type-specific antibody detectable by the delicate mouse protection test. That the Thomas strain is more effective is evident from the results of the preceding investigation (1) in which, in addition to the uniformly high degree of anti-Thomas potency in all sera, only 1 out of 10 rabbits failed to produce the small amount of antibody required to specifically protect mice against virulent Type III pneumococci. These facts strongly suggest that the Thomas bacteria are more effec-

tive in invoking an anti-Type III response in rabbits than are the typical Type III bacteria themselves. It seemed desirable, however,

TABLE I.

Comparison of the Response of Rabbits to the Injection of Typical Type III Pneumococci with the Response to Equal Amounts of the Thomas Strain.

Immune serum		Anti-Type III potency									
		Agglutination						Passive protection of mice *vs.* virulent Type III culture			
		Heated broth culture			Concentrated suspension of heated bacterial cells						
		Serum dilution			Serum dilution			Dose of culture, *cc.*			
Rabbit	Immunization material	Undiluted	2/5	1/5	Undiluted	1/5	1/10	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}
1	Thomas strain	++	+	0	fff	ff	f	D	S	S	S
2	Thomas strain	0	0	0	f	0	0	D	D–S	S	S
3	Typical Type III strain	0	0	0	0	0	0	D	D	D	D
4	Typical Type III strain	0	0	0	0	0	0	D	D	D	D
Anti-Type III horse serum (control)		+++	+++	+++	fff	fff	fff	D–S	S	S	S
Anti-Type I and anti-Type II horse serum (controls)		0	0	0	0	0	0	D	D	D	D

+++ = compact disc, with clear supernatant, after 2 hours at 37°C.; ++ = compact disc, but supernatant not entirely clear; + = definite granulation of the bacteria which persists after thorough shaking.

fff = sedimentation of agglutinated particles almost complete within 10 minutes at 25°C.; ff = sedimentation begun and fluid partially cleared by aggregation of agglutinated particles; f = definite granulation of the bacteria but fluid not cleared by aggregation.

D = mice died within 72 hours in all tests; D–S = protection irregular, mice dying in some tests and surviving in others; S = mice survived in all tests. Virulence controls died within 72 hours when injected with 1×10^{-8} cc. of culture.

that a comparison be made by experiments in which the amount of bacterial antigen and the number of injections were exactly the same.

Two rabbits were immunized against typical Type III pneumococci by two courses of six daily injections of heat-killed bacterial suspension equivalent to 5 cc.

of broth culture in each dose; a week's rest was given between the first and second courses, and the animals were bled 10 days after the last injection. Two other rabbits of the same weight were immunized with the Thomas strain by the same number of injections and with the same amount of bacterial suspension. A summary of the tests of the immune sera is presented in Table I.

The results in Table I show that the particular rabbits injected with typical Type III pneumococci in this experiment failed to produce antibodies reactive with the homologous bacteria, although both of the rabbits immunized with the same amount of the related but different strain, produced antibodies reactive with typical Type III pneumococci. While the anti-Type III potency of the anti-Type III and anti-Thomas serum as obtained from different immune rabbits will vary, the individual rabbits of each series used in this particular experiment yielded serum that can be accepted as possessing about the average potency exhibited by Tillett's series of 28 rabbits and by our previous series of 10 rabbits. Hence, with the above results considered as representative of those most likely to be obtained with rabbits, it is evident that rabbits can produce antibodies specifically reactive with typical Type III pneumococci more readily when injected with the Thomas strain than when injected with typical Type III pneumococci themselves. The results in Table I are important as evidence that this relation holds true when the amount of total bacterial antigen and the conditions of immunization are kept constant.

Both of the rabbits injected with the Thomas bacteria yielded serum potent against the homologous strain, while neither of the anti-Type III rabbit sera reacted at all with the Thomas strain. Since our chief interest is in the anti-Type III response, the results of the tests of the anti-Thomas potency are not included in Table I.

Comparison of the Active Immunity Response of Mice to Vaccination with Typical Type III Pneumococci with the Response to Vaccination with the Thomas Strain of Pneumococci.

The preceding experiment dealt with the responses of rabbits to immunization with typical Type III pneumococci and to immunization with the Thomas strain. The following experiment deals with the responses of mice to immunization with these two related but different kinds of pneumococci.

Immunization.—Two series of 60 white mice, about 3 months old, were selected for immunization; one series was injected with the Thomas strain and the other with a typical Type III strain. The organisms from 10 hour broth cultures were resuspended in salt solution and heated at 60°C. for 30 minutes. The suspensions of the two kinds of pneumococci were compared and when not approximately equal in turbidity, the stronger suspension was diluted until the concentration of total bacterial substance in each was about the same. Each mouse received, subcutaneously, 0.5 cc. of the respective vaccine (Type III or Thomas), equivalent to 0.15 cc. of broth culture, every 2 days for six doses; after which time one dose was given intraperitoneally. 10 days after the last injection, 20 mice from each series were tested for immunity against the two kinds of pneumococci. The remainder were given a second course of immunization with freshly prepared vaccine in the same doses as in the first course. After a rest period of 10 days, another lot of the immunized mice was tested for immunity either against the Thomas strain or against the typical Type III strain.

Tests of Immunity.—The active immunity of the vaccinated mice was tested by intraperitoneal injection of 0.5 cc. of broth containing the desired amount of a 10 hour broth culture of virulent pneumococci. Some of the mice vaccinated with typical Type III were tested against the Thomas strain and others against the homologous typical Type III strain; the Thomas-vaccinated mice were likewise tested against the typical Type III strain as well as against the homologous strain.

The doses used were 1×10^{-5} and 1×10^{-6} cc. of broth culture. These doses are not unreasonably small since both cultures were highly virulent and killed mice regularly in doses of 1×10^{-8} cc. Moreover, it seemed unwise to overtax the active immunity of the mice, especially in the case of Type III, in which the passive immunity conferred by immune horse serum is usually overwhelmed by doses above 1×10^{-4} cc. As a matter of fact, however, the Type III-vaccinated mice which had responded at all, apparently were able to resist doses approximately equivalent to those against which Type III horse serum can protect, for 3 out of 4 vaccinated mice which were tested against 1×10^{-4} cc. resisted this large dose in a later experiment.

The tests were made after both one and two courses of vaccinations. However, one course proved as effective as two courses, and since there is no essential difference in the tests, the results of the entire experiment are summarized in Table II.

Table II presents a summary of the results of experiments upon the active immunity response of mice to vaccination with the two related, but different, kinds of pneumococci—*i.e.*, a typical Type III strain and the Thomas strain. In a previous investigation (4) on the active immunization of mice against Type II pneumococci, it was found that within the zone of reasonably small dosage, the number of invading bacteria was a relatively unimportant factor in com-

parison to the factor of the differences in the immunity responses of the individual mice. For this reason, we believe the results of experiments on the active immunity response of mice can best be analyzed upon the basis of the percentage of individuals protected among a large group of vaccinated mice.

In the analysis of Table II, it is best to consider first the response of the mice as indicated by their immunity against the same strain as that with which they were vaccinated. It is evident that the mice vaccinated with Type III pneumococci responded fairly well in respect to the development of an immunity affording homologous protection, for 75 per cent of the 20 animals tested survived the injection

TABLE II.

Summary of Tests of the Response of Mice to Immunization with the Typical Type III Strain and with the Thomas Strain as Evidenced by Their Active Immunity against Virulent Cultures of These Two Kinds of Pneumococci.

Vaccinated mice	Mice vaccinated with typical Type III pneumococci		Mice vaccinated with Thomas strain of pneumococci	
	Homologous protection (*vs.* 1×10^{-5} or 1×10^{-6} cc. of virulent Type III culture)	Heterologous protection (*vs.* 1×10^{-5} or 1×10^{-6} cc. of virulent Thomas culture)	Homologous protection (*vs.* 1×10^{-5} or 1×10^{-6} cc. of virulent Thomas culture)	Heterologous protection (*vs.* 1×10^{-5} or 1×10^{-6} cc. of virulent Type III culture)
Number tested	20	15	22	15
Number protected	15	0	9	0
Percentage of protection	75	0	40	0

of virulent Type III bacteria. The response of the mice to the Thomas vaccination was apparently somewhat less effective, for only 40 per cent of the animals tested survived the injection of the Thomas or homologous bacteria. Thus, it is evident that from the standpoint of the development of immunity against the homologous kind of pneumococci, mice differ from rabbits in respect to the relative effectiveness of the typical Type III antigen and the Thomas antigen. Rabbits almost invariably respond well to the Thomas strain by producing immune sera of a high degree of potency, comparable in all respects to that elicited by Type II pneumococci, while an effective response of rabbits to the typical Type III antigen is the excep-

tion rather than the rule. The important fact in regard to the homologous protection in Table II is not that the mice do not respond uniformly well to the Thomas vaccination, but rather that such a large percentage of the mice are effectively protected by the Type III vaccination. The percentage of homologous protection exhibited by the Thomas-vaccinated group is just as high as that obtained in a previous investigation with Type II pneumococci, in which only 30 to 40 per cent of a large number of mice were effectively protected by homologous vaccination by the procedure employed in the present study. Thus, it appears that in respect to the development of homologous protection, mice respond just as well, and even better, to the Type III antigen than they do to two other kinds of pneumococci which in rabbits invoke incomparably better responses than do Type III pneumococci.

The response of the mice as evidenced by their immunity against the heterologous strain is less important, although it is interesting to observe that the mice failed to develop any detectable immunity against the related strain. If the mice had been more uniformly protected against the homologous strain, the lack of immunity against the heterologous strain would be important, for Type III immunization of horses does give antibodies reactive with the Thomas strain, and Thomas immunization of rabbits usually gives antibodies reactive with typical Type III strains.

Immunity Response of Mice as Evidenced by Passive Protection Experiments.

Since active immunity is not always the same as passive immunity, it seemed important to determine whether or not the serum of the vaccinated mice could confer passive protection to other mice.

In order to eliminate the factor of differences in the individual responses of the vaccinated mice, the serum from 8 mice vaccinated with Thomas bacteria was pooled and used as the anti-Thomas immune mouse serum; and the serum from 8 mice vaccinated with the Type III bacteria was used collectively as the anti-Type III immune mouse serum. Passive protection experiments were then made by the usual procedure with doses of 1×10^{-5} and 1×10^{-6} cc. of culture, the anti-Thomas serum being tested against both Thomas and Type III bacteria and the anti-Type III serum against both Type III and Thomas bacteria.

The results of the passive protection experiments were in all respects analogous to those obtained in the previously described active immunity tests. The factor of differences in the individual responses of the vaccinated mice was eliminated by pooling the immune serum. The anti-Type III immune mouse serum gave passive protection against Type III alone and not against the related Thomas bacteria; and the anti-Thomas immune serum gave protection against the Thomas bacteria and not against the typical Type III strain. The immune mouse sera, however, are scarcely comparable in homologous potency to the anti-Type III horse serum or the anti-Thomas rabbit serum; and hence their lack of heterologous potency is of less importance, for it is common experience that an antiserum of a slight degree of homologous potency is more usually of a higher degree of specificity.

Tests with a Recently Isolated Strain of Typical Type III Pneumococcus of Different Origin from the Type III Strain Employed in Vaccination of the Mice.

In the preceding experiments, the mice were tested against the same strain of typical Type III pneumococci as that employed in the vaccination. In the previous investigation (1), the immunological relationships evidenced in rabbit and horse antisera between this strain of Type III pneumococci and the Thomas strain were proved to hold true for three other strains of Type III pneumococci which had been recently isolated in Nashville. While there was this evidence in favor of the immunological homogeneity of typical Type III strains, it seemed important to repeat the experiments with a Type III strain known to be of different origin from the Rockefeller strain with which the mice had been vaccinated. A strain of Type III pneumococci which had been isolated about 10 days previously from a patient in the Vanderbilt University Hospital was utilized for this purpose in active immunity tests against mice vaccinated with the Rockefeller Type III strain and against mice vaccinated with the Thomas strain. Passive protection tests were also made as described previously with both anti-Type III and anti-Thomas immune mouse serum.

The results were exactly the same as those reported for the tests with the strain which had been used in vaccination. The mice vac-

cinated with the Rockefeller Type III strain were protected and the mice vaccinated with the Thomas strain were not protected against the Nashville Type III strain. Similarly, the immune serum from mice vaccinated with the Rockefeller Type III strain did give, and the serum from the mice vaccinated with the Thomas strain did not give passive protection to other mice against the Nashville Type III strain.

These tests were made against only the one foreign strain and are not presented as an argument for the homogeneity of the Type III group of pneumococci. But the identical results obtained in these experiments together with those in the preceding investigation, prove that the failure of reciprocal protection of mice against Type III pneumococci by active or passive immunization with the Thomas strain, and the failure of reciprocal protection of mice against the Thomas strain by active or passive immunization with the Rockefeller typical Type III strain, are not phenomena dependent upon the use of precisely the same Type III strain in the immunization and subsequent immunity tests.

Virulence of the Thomas Strain and of the Typical Type III Strain for Rabbits.

In view of the marked differences between the relative immunity responses of mice and those of rabbits to immunization with typical Type III strains and the Thomas strain, it is important to record the tests of the virulence of these strains for the two species of animals. Both the Thomas strain and the typical Type III strain (A 66, Hospital of The Rockefeller Institute) were highly virulent for mice, killing them regularly when doses of 1×10^{-8} cc. of broth culture were injected; but it has been shown by Tillett (5) that the virulence of Type III pneumococci for mice is by no means an index of the virulence of the same strains for rabbits.

Because of the probably marked differences in the natural immunity of different individual rabbits which has been suggested by Tillett, four rabbits were tested with each strain. The individual doses were 1 cc. intravenously, 5 cc. intravenously, 5 cc. intraperitoneally and 10 cc. intraperitoneally. None of the rabbits died even from the largest doses of the typical Type III strain. Two of the rabbits (those injected with 5 cc. and with 10 cc. intraperitoneally) were killed by the Thomas strain. The cultures injected had not been passed through rabbits.

From these results, it is evident that, although neither strain is highly virulent (at least without exaltation of potential virulence by rabbit passage), the Thomas strain is more virulent for rabbits than is the typical Type III strain.

DISCUSSION.

The preceding experiments have dealt with the relative effectiveness of the responses of mice and of rabbits to immunization with a typical Type III strain in comparison with their response to immunization with the Thomas strain which is related to but not identical with Type III pneumococcus.

In regard to the response of rabbits, the apparent relationship between the antigenic effectiveness of the two kinds of pneumococci was the same as that already described in the preceding paper; but the results of the present study are important in that the conditions of immunization and the dosage were kept constant in the animals injected with the two strains. From the standpoint of the development of homologous, specific antibodies, the Thomas strain was incomparably more effective; the anti-Thomas rabbit sera having, as usual, a high degree of anti-Thomas potency; and the anti-Type III rabbit sera being, as is frequently the case, devoid of anti-Type III potency. The greater effectiveness in rabbits of the Thomas antigen was also true from the standpoint of anti-Type III potency; and the results in this investigation, as in the preceding one, furnish evidence that rabbits can produce antibodies specifically reactive with typical Type III pneumococci, more readily when injected with the Thomas strain than when injected with typical Type III pneumococci themselves.

With mice, the results were quite different. In the experiments with these animals, it was found that vaccination with typical Type III pneumococci elicited somewhat better immunity responses,—as indicated by the higher percentage of individuals which were protected,—than did vaccination with the Thomas strain. The immunity in both instances was limited to homologous protection, for both the Thomas strain (which in rabbits invoked an anti-Type III response) and the typical Type III strain (which in horses invokes an anti-Thomas response) failed to invoke an immunity against the related but different kind of Pneumococcus. From the standpoint of the

active immunity exemplified by homologous protection, the typical Type III strain proved not only to be more effective antigenically than was the Thomas strain, but also, in comparison with the results of a previous study (4), it appeared to be a more effective antigen in mice than are Type II pneumococci,—although the latter, like the Thomas pneumococci, almost invariably give better immunity responses in rabbits.

Thus, the results show that the relative antigenic effectiveness of these two kinds of related pneumococci, is different in different animals; in rabbits, the Thomas strain is more effective than typical Type III; in mice, the typical Type III is better than Thomas. These relationships furnish an example of the influence of the species of animal upon the apparent effectiveness of bacterial antigens, for while the potential antigenicity is resident in the chemical structure of the antigen, its actual effectiveness in practice is determined by the individual response of the particular animal. This example of the marked differences in the selective effectiveness of the two different antigens is the more interesting in view of the close serological relationship of the two kinds of pneumococci utilized as antigens.

SUMMARY.

The paper reports an experimental comparison of the antibody responses of mice and of rabbits to immunization with typical Type III pneumococci and a strain related to Type III. The results as a whole show that the relative antigenic effectiveness of these two kinds of related pneumococci, is different in different animals. In rabbits the strain related to but not identical with Type III elicits the better response; in mice, the typical Type III strain is more effective. These relationships furnish an example of the influence of the species of animal upon the effectiveness of even closely related bacterial antigens.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

I. Spontaneous and Experimental Udder Infection.

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During the past four decades many outbreaks of scarlet fever attributed to contamination of milk supplies have been reported. This occurrence claimed considerable attention during the later '80's of the 19th century. Since then, although outbreaks attributed to milk have been recorded, the number has apparently declined. In certain particulars epidemics due to contaminated milk resemble each other. Their explosive nature—the bulk of the cases occur during a short period, usually within a week—is characteristic. The history that the sick have partaken of milk from a common source and the fact that the disease is not epidemic in other parts of the community having a different milk supply are equally significant.

The origin of milk-borne epidemics of scarlet fever has in the past led to much discussion.

Probably the most commonly held view is that of actual contamination of the milk by convalescents or persons actually sick with the disease. This view is supported by many recorded outbreaks in which persons known to have come in contact with scarlet fever or who were actually suffering from the disease milked the cows, mixed, handled, or bottled the milk. Among others, Hemenway (1) records a large epidemic attributed to milk thus contaminated. Other outbreaks regarded as milk-borne could be traced to actual cases who delivered the milk from door to door. Chalmers (2) reports such an outbreak. In addition the return of milk bottles from houses where scarlet fever was present was held responsible for the disease along particular milk routes.

The widespread and severe nature of certain outbreaks in England during the period from 1880 to 1900 led to investigations that directed attention to the cow as a source of infection. The difficulty of tracing human contamination strength-

ened the suspicion. Power (3) and Klein (4) as early as 1882 attributed one outbreak to the contamination of milk from a case of puerperal fever in a cow. Klein showed that cows inoculated subcutaneously with material obtained from the throats of human patients developed abscesses at the injection sites. Purulent material from such abscesses when injected into healthy cows also produced abscesses. Later Power (5), Cameron (6), and Klein (7) reported their findings in the Hendon outbreak in which they failed to find a human source of infection. Suspicion was directed to certain newly purchased cows suffering with a malady of the skin of the udder and teats. This they regarded as a specific infection transmitted by the hands of the milker. The condition was characterized by general constitutional disturbance, sore throat, discharge from the eyes and nose, vesicular eruptions of the skin of the buttocks and udder. They regarded the rupture of the teat vesicles as the source of the milk inoculation. Russell (8) encountered a similar outbreak in which 101 persons contracted scarlet fever; a malady similar to the Hendon disease affecting two cows was noted in the herd. A calf fed the milk of one of these developed a severe febrile reaction. Hill (9) likewise reported milk-borne scarlet fever; certain cows in the dairy from which the supply was drawn were found affected with Hendon disease. Hamar and Jones (10) also cited an outbreak in which a disease of the skin of cows similar to that noted by Power, Klein, and Cameron was present in the herd. They were inclined to attribute the human infection to the cows since the milk was known to be infective before human cases occurred on the farm. M'Fadyean (11) disagreed with them on the grounds that Hendon disease as a scarlatinal infection of cows is unproved and that the milker in whose family scarlet fever occurred may have been responsible for the epidemic through contamination. As a further criticism that Hendon disease is not a specific scarlatinal infection of cows he points out that although the disease was first recognized in 1882 and seemed to prevail for a year or two it had not attracted further attention until 1909 when a similar disease was reported.

It is apparent then that there are several views regarding the method by which milk may become infective: first, contamination of the milk during milking or handling; second, the return of contaminated bottles or utensils from the household in which the disease exists; and third, a scarlatinal disease of the skin and udder of cows from which discharges may enter the milk during milking.

With the change in the status of the streptococcus as the etiological agent of scarlet fever during the past few years, a fourth means of contamination becomes apparent. The findings of Savage (12), T. Smith and J. H. Brown (13), Davis and Capps (14), Brown and Orcutt (15), and Benson and Sears (16) in milk-borne epidemics of septic sore throat are of considerable interest in this connection. It

has been shown that *Streptococcus epidemicus* may be implanted in the udder and be shed in the milk in such numbers as to give rise to severe outbreaks of sore throat among those consuming the milk. That such is not unlikely in outbreaks of scarlet fever attributed to milk seems plausible. This is especially significant in many epidemics traced to dairies where no mention is made of clinical examination of the udder or bacteriological examination of the milk. The possibility of udder infection may have been overlooked.

Our problem concerns itself with udder infections with streptococci similar to those found in scarlet fever.

History of a Milk-Borne Epidemic of Scarlet Fever.

Through the courtesy of the New Jersey State Department of Health we learned of a sharp outbreak of scarlet fever in a small town. About 200 cases occurred. Of these 159 developed from May 20 to 25, 1927. The State authorities found that the bulk of the cases was confined to a certain milk route supplied by one distributor. Further information directed their attention to one of the farms supplying the dairy. On this farm a daughter had scarlet fever in March, 1927. In addition a young man employed as milker had visited his home shortly before the outbreak where there was a child sick of scarlet fever. Representatives of the State Department of Health made throat cultures from everyone suspected of contaminating the milk and obtained hemolytic streptococci in two instances from throats of men handling the milk at the distributor's. For these cultures we are indebted to Mr. J. V. Mulcahy, Chief of the Bureau of Bacteriology of the State Health Department. In addition he furnished us with a culture from the young man who milked the cows, but it was of the *viridans* type.

On May 25, Dr. I. H. Shaw, veterinarian for the State Department of Health, visited the farm and examined microscopically the milk sediment from each cow. He noted one chronic case of mastitis (Cow 11) and an acute injury of the teat of the left hind quarter (Cow 3). The milk sediment of Cow 3 contained leucocytes and cocci.

In the meantime the milk from this farm had been excluded from the supply and a pasteurizer installed in the distributor's with the

result that the epidemic rapidly subsided. At our suggestion, and through the courtesy of the State Department of Health, the farm was visited on June 4, 1927. Samples were drawn directly from the udder of each cow into separate sterile bottles.

Examination of Milk from Each Cow.

The samples were obtained on the evening of June 4, refrigerated at once, and plated late the same night. The normal appearing milk was plated in two dilutions, 1:20 and 1:100; that from abnormal quarters at 1:1,000 as well as the lower dilutions. All plates were prepared with 0.5 cc. of defibrinated horse blood and 10 or 12 cc. of melted agar. Thirteen cows comprised the herd. The udders of eleven were normal. The plate cultures revealed nothing of significance. Cow 11 had chronic mastitis of the left hind and right fore quarters. The plate cultures revealed non-hemolytic streptococci in enormous numbers both in the involved and apparently normal quarters. Cow 3 had a severe involvement of the left hind quarter. The teat had been injured, the quarter was swollen and could be milked with difficulty. The milk was yellow and of the consistency of heavy cream. All the blood in the plate containing as little as 1:1,000 cc. of milk was hemolyzed within 12 hours. The centrifuged sample revealed a large quantity of sediment consisting of packed masses of leucocytes and enormous numbers of short chained streptococci. After refrigeration for 12 hours the milk was further diluted and plated so that finally it was possible to estimate that it contained 345,000,000 streptococci per cc. Colonies sufficiently isolated were chosen for subculture. It may be said that the initial tests, such as those for the presence of capsules, the laking of blood in the test-tube, pathogenicity for rabbits, and a final pH of 5.0 in dextrose broth, indicated that this culture was of human origin. In addition to the human type a small proportion of the non-hemolytic bovine streptococci was found in the original sample.

The Spontaneous and Experimental Infection in the Cow.

Cow 3 was purchased by this Department and is hereafter referred to as No. 1452. 4 days after the first observation the quarter was swollen, firm, and the teat showed a healing scar. Yellow, purulent milk could be expressed only with difficulty. Bacteriological examination revealed relatively few hemolytic streptococci and enormous numbers of the non-hemolytic mastitis type. Evidently the bovine type noted June 4 had nearly replaced the hemolytic streptococcus. The inflammation continued in the quarter until the cow was slaughtered on June 23. During this time hemolytic streptococci were always present in small numbers and the mastitis type in enormous numbers.

On one occasion a single colony of the hemolytic type was obtained from the milk of the right hind quarter. This strain was identical in all respects with that obtained from the left hind quarter. Although the milk from the other quarters was plated frequently streptococci were not found.

On June 9, the right hind quarter was inoculated by means of a teat tube with 1/1,000,000 cc. of a 24 hour broth culture of the hemolytic streptococcus from the left hind quarter. No reaction occurred and examination of the milk during the next 3 days failed to show the organism.

On June 14, the right fore quarter was inoculated by means of a teat tube with 1/500,000 cc. of the hemolytic culture and the right hind quarter with 1/100,000 cc. of the same culture. 7 hours later the streptococcus could not be cultivated from the milk from either quarter. After 24 hours there was little to be detected clinically. The appearance of the milk was not greatly altered, and the quantity of sediment was not excessive but it contained leucocytes and a few diplococci. Plate cultures, however, revealed 25,600 hemolytic streptococci per cc. in the milk from the right hind quarter and over 1,000,000 per cc. in that from the right fore quarter. After 48 hours the right hind quarter was hot, the milk was yellow and contained large, irregular floccules. The right fore quarter revealed nothing abnormal except that the milk was yellow and flocculent. Samples from both quarters revealed an excessive amount of sediment composed of leucocytes and streptococci. The bacterial counts of the milk were: right fore quarter = 45,000,000 hemolytic streptococci per cc., and right hind quarter = 1,240,000 per cc. The next day 38,000,000 hemolytic streptococci per cc. were found in the milk from the right fore quarter and 1,240,000 per cc. in that from the right hind quarter. From this time onward the number began to decline until on the 14th day the milk from the right fore quarter failed to show streptococci while that from the right hind quarter revealed 26,000 per cc.

During the period of observation it can be said that the inoculation failed to produce well defined clinical disturbances in the inoculated quarters, although the milk was purulent.

The udder obtained from the abattoir at the time of slaughter revealed a pronounced atrophy of the left hind quarter characterized by severe degeneration of the secreting structures and hyperplasia of the interstitial tissue. The two inoculated quarters showed lesions of the mucosa of the lower portion of the udder involving the mucosa of the large milk ducts accompanied by a purulent exudate.

Since the spontaneous case of infection was complicated by injury and secondary infection with the usual type of mastitis streptococcus,

the question might be raised as to whether the factor of injury is a necessary precursor of infection. Further the experimental disease incited by inoculation failed to resemble the spontaneous disease. In order to throw more light on these questions a second series of inoculations was made.

Cow 1462.—A Holstein cow was injected by means of a teat tube inserted into the left hind quarter with 1/1,000,000 cc. of an 18 hour serum broth culture of the 6th transfer of the streptococcus obtained from the left hind quarter of Cow 1452. Plate cultures prepared from similar dilutions indicated that between 15 and 20 streptococci were injected. Plate cultures of the milk 7 hours after injection failed to show streptococci. 24 hours after injection the quarter still appeared normal. The milk contained only a little sediment composed of a few leucocytes and round cells and a few diplococci. The plate culture revealed 115,000 hemolytic streptococci per cc. After 31 hours the quarter was swollen, tense, hot, and

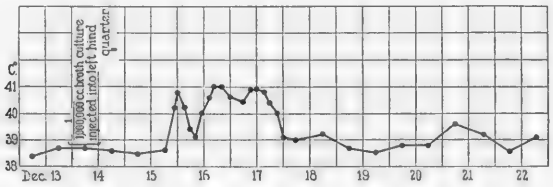
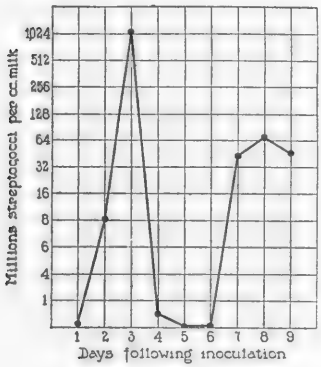


CHART 1. Temperature reaction, Cow 1462, following injection of left hind quarter.

painful. The milk contained an excess of fat but the amount of sediment was not excessive. There were 2,000,000 streptococci per cc. At 36 hours the quarter was greatly swollen. The milk was yellow and serous and contained large floccules. It coagulated on boiling. Plates revealed 8,320,000 streptococci per cc. 48 hours after injection there was a severe systemic reaction characterized by chills, fever (Chart 1), and depression. Swelling of the quarter was pronounced. The milk was seropurulent. It was estimated that 1,200,000,000 streptococci per cc. were being eliminated. On the 3rd day the quarter was distinctly reddened, the milk purulent, and 510,000 streptococci per cc. were recorded. A blood culture was negative. The cow had a fever. The reddening of the skin spread to the other quarters on the 4th day, persisted throughout the 5th day, and began to subside on the 6th day. It had disappeared by the 7th day. During this period the number of streptococci decreased until a minimum of 20,000 per cc. was reached. However they increased during the 7th, 8th, and 9th days, reaching the high point of 69,000,000 on the 8th day. There was a corresponding rise of temperature on the 7th and 8th days (Chart 1). By the 13th day their number

had declined to 2,000,000. From this time the acute inflammation gradually subsided with gradual atrophy and with a further decline in the secretion until on the 47th day only 25 or 30 cc. of purulent milk was obtained. Hemolytic streptococci were still present on this day. Chart 2 illustrates the number of streptococci per cc. of milk from the left hind quarter of Cow 1462 for 9 days following the inoculation.

It is to be noted that during the height of the attack the general reaction was severe, milk secretion was almost entirely suppressed, a fall from 8 pounds per milking to less than 1 pound being recorded.



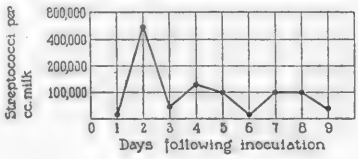


CHART 2. CHART 3.

CHART 2. Streptococci per cc. of milk during the first 9 days subsequent to injection of left hind quarter of Cow 1462.

CHART 3. Streptococci per cc. of milk during the first 9 days after injection of right hind quarter of Cow 1462.

27 days after the left hind quarter had been artificially infected one-millionth cc. of broth culture was instilled into the lower cistern of the right hind quarter. It was estimated that approximately 70 streptococci were injected. 7 hours later there was nothing in the appearance of the milk to arouse suspicion and 1 cc. failed to reveal the streptococcus. After 24 hours the milk appeared normal. The sediment contained a few leucocytes and plate cultures revealed 1,800 streptococci per cc. 2 days following the inoculation the quarter failed to show abnormalities. The milk was yellow and thickened; when centrifuged the sediment comprised about 1/20 of the total volume. It was composed of masses of leucocytes, diplococci, and short chained streptococci. The maximum count of 556,000 streptococci per cc. was reached on this day. During the next 9 days the udder and milk were examined daily. Lesions of the udder were not detected

clinically. The milk was always purulent and the sediment at times made up 1/10 of the volume. The number of streptococci varied from day to day, the minimum count recorded during this period being 18,000 and the maximum 136,000 per cc. On the 21st day the milk was still purulent and flocculent and contained 20,000 streptococci per cc. Chart 3 is included for comparison with Chart 2. It indicates the number of hemolytic streptococci eliminated per cc. during the first 9 days following inoculation of the right hind quarter. The two charts afford a sharp numerical contrast. In the primary inoculation the maximum of over 1 billion was reached on the 3rd day following injection, with a rapid fall until the 6th day and a subsequent sharp rise on the 7th, 8th, and 9th days. Chart 3 illustrates the elimination of streptococci subsequent to injection of the right hind quarter. Here the peak of 550,000 was reached on the 2nd day with a sharp decline on the 3rd day and irregularity thereafter.

It is true then that the streptococcus isolated from the spontaneous infection was of sufficient virulence in spite of cultivation for 6 months on artificial media to incite a severe mastitis. During the acute inflammation there were marked systemic disturbances characterized by fever, increased pulse and respiratory rates, congestion of the conjunctiva, inappetence, suppression of milk, and stiffness of the joints. We regarded the animal as critically ill during this period.

In data from both the spontaneously infected and the experimental cow evidence exists that the primary attack so increased the general resistance that the secondary injection resulted in entirely different manifestations. Here only mild local disease resulted; although the streptococcus established itself, its multiplication never reached the maximum recorded in the primary attack.

It may be argued that the spontaneous case (No. 1452) referred to is an isolated example of such infection.* While this is true to a certain

* While these data were being assembled, Mr. Friend Lee Mickle, Director of the Bureau of Laboratories, Connecticut State Department of Health, sent three cultures of streptococci for examination. The interest of the Health Department had been aroused because of an outbreak of mild scarlet fever which appeared among the customers along a certain milk route. One of the cultures was isolated from the udder of a cow in the herd supplying the milk, another from the throat of the owner of the herd, and a third from a case of scarlet fever on the milk route. These proved similar in cultural characters, pathogenic properties, and antigenic affinities to those obtained by us from the udders of two cows. In all probability the particulars of this outbreak will be published by the Connecticut State Department of Health.

extent, nevertheless material obtained from another case of mastitis associated with a similar organism suggests that such infections may occur at any time. Although at the time of isolation the significance of the bacteriological findings in this spontaneous case was not realized, nevertheless the organism was regarded as a human type other than *S. epidemicus*. A brief statement concerning this case follows.

Cow 4262.—Milk drawn from all four quarters into a sterile bottle May 11, 1925. When plated it was found to contain 720 colonies per cc.; 25 per cent were large zoned, hemolytic colonies. May 19, 1925, mastitis of the right fore quarter was noted. The milk was purulent and flocculent. 77,000 hemolytic colonies per cc. were recorded. On May 22 and 23, the milk from all quarters was examined with negative results. The hemolytic streptococcus had been replaced by the bovine non-hemolytic type. The milk was again examined on May 27 and 28, and the later findings confirmed. Little of significance could be obtained from the history of this cow except that it had been in the herd for 3 years. During the preceding lactation periods many attacks of mastitis had been noted. During the 2 months prior to our examination four attacks of mastitis of the right fore quarter had been reported. The hemolytic streptococcus was definitely of the human type as proved by the usual tests and, as it will be shown later, closely resembled those isolated from Cows 1452 and 1462. No record is available which indicates that this animal was responsible for an outbreak of scarlet fever.

DISCUSSION.

Heretofore contamination has been regarded as the usual means of spread of scarlet fever by milk. The findings in regard to septic sore throat, however, indicate that udder infection with the human streptococcus is far more likely. The same may be said of milk-borne epidemics of scarlet fever.

Unfortunately our examination was conducted after the epidemic had subsided. It is true though that when the milk from this farm was withheld from the general supply and the general supply pasteurized the outbreak subsided. When we made our examination the owner maintained that the milk from the left hind quarter of the infected cow was "all right" although he had withheld it from the general supply. It can be argued, however, that such milk was fully capable of causing severe illness among the consumers provided it entered the general supply. If 1 quart of milk drawn at the time when the streptococci were most numerous was mixed with the herd supply, the actual

dilution in this instance would amount to 1:100 since there were 12 cows contributing about 100 quarts. Our maximum count indicated well over 300 million per cc. in the spontaneous case and over 1 billion in the experimental inoculation. Assuming that the infective product was again diluted at least a hundred times at the distributor's the number of streptococci in even a small quantity of milk would be relatively high.

Spontaneous Case 1452 and experimental Case 1462 are examples of extremely severe infections and they indicate that the organism was highly virulent for the cow. The infection obliterated for practical purposes the primarily involved quarter. However the resulting general resistance in both instances was insufficient to protect other portions of the udder from infection. This argues for a prompt exclusion from the milking shed of cows with involved quarters since the organism may gain entrance to normal quadrants without exciting severe reactions.

In sharp contrast to these cases is that of Cow 4262 in which the organism was known to be present in the milk on two occasions 8 days apart. It probably inhabited the udder during this period. However it disappeared and was replaced by a bovine type and was not found subsequently. That this cow failed to cause trouble to consumers can be explained on several grounds, (1) that the milk contained relatively few organisms, (2) that the milk was mixed with that of a large number of cows (50 or 100) and sold in a large city where a few cases of scarlet fever would attract little attention, and (3) that when the milk became abnormal it was eliminated from the supply. This case argues for a prompt investigation of the milk of all cows when milk-borne infection is suspected, since a few days delay in instances of this kind may be sufficient for the disappearance of the streptococcus from the udder.

The period of incubation is of considerable practical importance. In regard to this period little is known in spontaneous infection. In all the experimental inoculations there was little to arouse suspicion during the first 24 hours although streptococci could be readily detected in the milk. After this time abnormalities in the appearance of the milk were apparent. It must be borne in mind that in these experiments the organisms were introduced into the milk cistern, and

although the number of streptococci administered was small, in all probability in the spontaneous disease the infective dose would be even smaller; and unless contaminated material was introduced by teat tubes or other means the organisms would probably be deposited at the meatus or about the ends of the teats. This indicates a somewhat longer incubation period than that observed in the experiments.

SUMMARY.

The clinical and bacteriological findings in two cows the udders of which became infected under natural conditions with hemolytic streptococci of the scarlet fever type are discussed. One of the cows was found in a herd supplying raw milk to a small town where a milk-borne outbreak of scarlet fever had occurred a short time before. When small numbers of the streptococcus obtained from this case were injected into the udder of a normal cow severe mastitis accompanied by a well marked general reaction resulted. Evidence leads to the conclusion that a severe attack of mastitis due to this organism in one quarter does not sufficiently immunize the other quarters to protect them completely since the streptococcus can be readily implanted in them. The secondary infections were much milder than the original process.

We wish to acknolwedge our indebtedness to Mr. W. T. Eakins, Assistant Epidemiologist of the New Jersey State Department of Health, who furnished us with the history of the outbreak; also to Mr. J. V. Mulcahy who supplied us with cultures F. C. and M. B. obtained from the throats of milk handlers, and to Dr. I. H. Shaw for accompanying us to the farm and for other courtesies.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

II. A Study of the Scarlet Fever Type of Streptococci Isolated from the Udder of the Cow.

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Our first paper (1) dealt with the clinical manifestations resulting from invasion of the udder with a hemolytic streptococcus of probable human origin. It was shown that the culture isolated from the udder was capable on inoculation into the udder of a second cow of producing severe mastitis.

It was also stated that the ability of the streptococcus to lake blood in the test-tube, its pathogenicity for rabbits, and a limiting hydrogen ion concentration of pH 5.0 in dextrose broth differentiated it from the bovine streptococcus and indicated its human origin. The present paper deals with this phase of the question and a further correlation of these streptococci with those of the scarlet fever group.

Source of Cultures.

The cultures were drawn from several sources. Our own isolations from the udder of the spontaneous and experimental cases were regarded as the primary type. Those strains kindly furnished us by Mr. J. V. Mulcahy, Chief of the Bureau of Bacteriology of the New Jersey State Department of Health, who isolated them from the throats of milk handlers at the dairy, have been indicated as F.C. and M.B. Two cultures of scarlet fever streptococci of known origin were used, one (N.Y.V) furnished by Dr. A. R. Dochez, of the College of Physicians and Surgeons, Columbia University, and the other (Sc. 55) supplied by Dr. Anna F. Williams of the Department of Health of the City of New York. In addition three cultures of *Streptococcus epidemicus* are included in some of the comparisons. Of this type, C. 54 is the second bovine passage strain of the streptococcus isolated by Brown and Orcutt (2) during a milk-borne epidemic of septic sore throat. The others (744 and 4560) were obtained at various times by one of us (F.S.J.) from the udder of cows.

Morphological and Cultural Findings.

It can be said that little difference exists between the organisms isolated from Cows 1452, 1462, 4262, and the throats of F. C. and M. B. and the known scarlet fever strains. In all, chains of cocci up to 30 in number are not unusual. All are Gram-positive and all encapsulated. On the whole the capsules are considerably smaller than those of *S. epidemicus*. After 24 hours incubation in plate cultures containing 0.5 cc. of defibrinated horse blood and 10 cc. of agar prepared from veal infusion the surface colonies are round,

TABLE I.

The Fermentation Characters of the Streptococci Isolated from the Udder and from the Throats of the Milk Handlers.

Culture	Final hydrogen ion concentration in							Action on milk
	Dextrose	Lactose	Saccharose	Mannitol	Raffinose	Inulin	Salicin	
Cow 1452, L.H.Q.	5.2	5.2	5.3	7.2	7.6	7.6	5.3	Firm coagulation.
" 1452, R.H.Q. (before inoculation)	5.2	5.2	5.2	7.2	7.6	7.6	5.4	" "
Cow 4262, R.F.Q.	5.0	5.0	5.0	7.4	7.4	7.6	5.3	" "
" 1462, L.H.Q.	5.2	5.4	5.3	7.6	7.4	7.4	5.6	" "
Throat F.C.	5.2	5.2	5.0	7.2	7.6	7.6	5.3	" "
" M.B.	5.2	5.3	5.3	7.4	7.4	7.6	5.4	" "
Non-hemolytic mastitis streptococcus from Cow 1452, L.H.Q.	4.5	4.6	4.6	7.0	7.6	7.6	4.7	" "

flattened, translucent, and rarely exceed 1 or 1.5 mm. in diameter. They are surrounded by a sharp clear zone. The deep colonies vary in shape from round to biconvex and form the nucleus of beta hemolytic zones averaging 2.5 mm. in diameter. A further 24 hours incubation fails to increase the size of the colonies to any great extent although the hemolytic areas are increased to 3.0–3.5 mm. The colonies of *S. epidemicus* employed in the comparisons were larger, more sharply raised, and distinctly mucoid. The hemolytic areas were larger. The non-hemolytic streptococcus isolated from the udder of Cow 1452 is included in Table I for purposes of comparison.

To test the biochemic activities, all cultures were grown in fermented broth (pH 7.4), to which sufficient of the test substance had been added to make a 1 per cent solution, for 7 days. The results are recorded in Table I.

Table I indicates a close resemblance in the fermentation characters of the streptococci isolated from the spontaneous udder in-

TABLE II.

The Effect of the Cultures on Rabbits.

Rabbit No.	Weight	Intravenous injection—24 hr. broth culture	Result
	gm.		
1	1,010	1 cc. Cow 1452, L.H.Q., Culture A	Died. 28 hrs. Septicemia
2	2,050	1 " " 1452, " " B	" during night of 2nd day. Septicemia
3	1,105	1 " " 1452, R.H.Q., before inoculation	Died. 36 hrs. Septicemia
4	1,425	1 cc. Cow 1452, L.H.Q.	" on 4th day. "
5	1,575	1 " " 4262, R.F.Q.	Febrile reaction during first 7 days. Abscess right hock. Chloroformed on 10th day. Streptococci recovered from abscess
6	1,040	1 " culture from throat of F.C.	Febrile reaction during first 2 days. Bacteriemia during first 24 hrs. Chloroformed on 11th day. Abscess right hock. Vegetation on right auriculoventricular valve
7	1,130	1 " " " " " M.B.	Febrile reaction throughout period Bacteriemia during first 3 days. Chloroformed on 11th day. Abscesses both elbows. 4 vegetations on right auriculoventricular valve

fections and those obtained from the throats of the handlers at the distributor's. All attacked dextrose, lactose, saccharose, salicin, and coagulated milk whereas the other substances were not fermented. It is of interest to note that the passage of strain Cow 1452 through the udder of Cow 1462 failed to change its fermentation characters. The acid production of the non-hemolytic bovine streptococcus offers a sharp quantitative comparison.

Mention has been made of the pathogenicity of our strains for rabbits. As soon as feasible after the isolations rabbits were injected intravenously with 1 cc. of a 24 hour broth culture. The results are recorded in Table II.

The cultures from Cows 1452 and 1462 were more virulent for rabbits than the others. That from Cow 4262 behaved about the same as did those isolated from the milk handlers. These streptococci tended to localize in the joints and heart valves.

It will be noted that the preceding characters indicate human origin, since the bovine streptococcus produces more acid and is not pathogenic for rabbits in similar doses. They afford no specific differentiation between *S. scarlatinæ and S. epidemicus*, although by inference it might be said that, since Culture 1452 was identical with those obtained from the throats of the milk handlers during an epidemic of scarlet fever, all three were of the scarlet fever type.

Further comparisons were regarded as essential. Attempts were made to group the strains by their agglutination affinities. Even though the cultures were grown for long periods at room temperature, as suggested by Shibley (3), and suspended in 0.2 per cent NaCl solution made alkaline with NaOH, the results were inconclusive.

The recent contribution of Lancefield (4) for the differentiation of streptococci by means of precipitin offered a means of separation. Lancefield has shown that immunization for long periods with broth cultures of streptococci results in the production of precipitin. For antigenic purposes in conducting the tests the growth from a liter of broth culture is suspended in salt solution to which sufficient N HCl is added to make a concentration of N/20. The suspensions are then extracted in a water bath at 100°C. for ¾ hour and neutralized with N NaOH. They are then freed of bacteria by centrifugation. When suitable quantities of such antigen are brought in contact with potent antiserum, the usual precipitin phenomena develop. When the fluids are mixed considerable precipitate is formed.

Rabbits were immunized by intraperitoneal injections of killed cultures followed by smaller doses of living organisms. For this purpose strains Cow 1452, F. C., Scarlet Fever N. Y. V, Scarlet Fever 55, and *S. epidemicus* Cow 4560 were used. Even after prolonged injection the sera of rabbits treated with strain Cow 1452 revealed only a weak precipitin. Scarlet Fever 55 failed to give rise to any

precipitin. A fair serum was obtained with N. Y. V and strong sera with F. C. and *S. epidemicus* 4560. In Table III we have recorded the reactions resulting from the addition of 0.15 cc. serum to 0.4 cc. antigen.

By means of Lancefield's procedure it was possible to divide the streptococci into two groups. Those associated with the milk-borne epidemic of scarlet fever—Cow 1452, Cow 1462, and F. C. and M. B.—

TABLE III.

Precipitation of Extracts of Streptococci with Various Antisera.

Streptococcic extract	Antisera			
	Rabbit immunized with			
	Culture Cow 1452	Throat culture F.C.	Scarlet fever streptococcus N.Y.V	*S. epidemicus* 4560
Cow 1452 L.H.Q.	+*	+++	+++	+−
" 1452 R.H.Q., before injection	+	+++	+++	+−
after injection	+	+++	++	+−
Cow 1462 L.H.Q.	+	+++	++	+−
Throat F.C.	+	+++	++	+−
" M.B.	+	+++	++	+−
Cow 4262	+	++	++	+−
Scarlet fever 55	+	+++	++	+−
" " N.Y.V	+	+++	++	+−
S. epidemicus Cow 54	−	−	+−	+++
" " " 744	−	−	+−	+++
" " " 4560	−	−	+−	+++

* + indicates moderate contact reaction with definite flocculation after mixing; ++, a well defined contact with considerable flocculent precipitation after mixing; +++, the maximum reaction.

behaved like the scarlet fever streptococci N. Y. V and 55. The culture isolated from Cow 4262 had a similar antigenic affinity. The three strains of *S. epidemicus* comprised a separate group. There was a slight tendency to precipitation, as indicated by the +− reactions, when the extracts from the scarlet fever group were treated with *S. epidemicus* serum, but for purposes of classification these can be ignored since the reactions indicated by ++ and +++ were so marked as to be unmistakable.

In the course of the further correlation it proved possible in two instances by means of intracutaneous tests on persons susceptible to scarlet fever toxin to show that culture Cow 1452 produced a toxin similar to that of scarlet fever streptococcus N. Y. V and that this toxin was neutralized by scarlet fever antitoxin.

The following protocols are submitted.

Cultures 1452 and N.Y.V were grown for 5 days in flasks containing a shallow layer of broth. The cultures were centrifuged and the supernatant fluid passed through Berkefeld candles of V porosity. Dr. Richard E. Shope of this Department, who had never had scarlet fever, volunteered as a subject. One of the writers (F.S.J.) also proved susceptible to the scarlet fever toxin. The filtrates were diluted in salt solution and the equivalent of 1/100 cc. of filtrate injected intradermally. The same amount of each filtrate was mixed with 0.05 cc. of scarlet fever antitoxin and injected. In all there were four injections, (1) scarlet fever filtrate, (2) scarlet fever filtrate + scarlet fever antitoxin, (3) culture Cow 1452 filtrate, and (4) culture Cow 1452 filtrate + scarlet fever antitoxin.

Subject R.E.S. responded severely at Sites 1 and 3. Site 2 was negative and Site 4 revealed only a slight reddening without swelling, the redness subsiding rapidly.

Subject F.S.J. responded most severely at Site 3, and had a definite reaction a Site 1. Sites 2 and 4 revealed some reddening which rapidly faded.

DISCUSSION.

The evidence seems clear that a streptococcus indistinguishable from the scarlet fever type may, under certain conditions, gain access to the cow's udder and there produce characteristic inflammatory changes.

That the streptococci isolated from the cows under study fulfill the criteria outlined for human streptococci is shown by their ability to lake blood in the test-tube, their final hydrogen ion concentration (5.0) in dextrose broth, and their pathogenicity for rabbits. Furthermore they may be differentiated from *S. epidemicus* by their antigenic complex when tested with specific precipitin. Extracts prepared in the manner described by Lancefield precipitated well with scarlet fever serum but failed to do so when mixed with *S. epidemicus* antiserum. The neutralization of the toxin of culture Cow 1452 by scarlet fever antitoxin is further evidence of similarity. As far as we could determine the passage through a second cow failed to markedly change the *S. scarlatinæ*-like character of the strains.

SUMMARY.

The streptococcus isolated from the udder of a cow on a farm where an outbreak of scarlet fever originated has been correlated with known scarlet fever strains. This streptococcus and another also isolated from the udder of a cow are indistinguishable in cultural characters and certain antigen affinities from *S. scarlatinæ*. Skin tests indicate that the strain isolated from the milk of the cow in a herd to which the scarlet fever epidemic was attributed produced a toxin which was neutralized with scarlet fever antitoxin.

We are indebted to Dr. Richard E. Shope of this Department for cooperation in making the skin tests.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

III. The Influence of Milk on the Growth of Scarlet Fever Streptococci.

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Plate 38.

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The view usually held covering milk-borne epidemics of scarlet fever is that the streptococci originate in the throats of the milk handlers and through contamination gain access to the supply. It is difficult to explain on such grounds the heavy incidence of infection among the consumers of pooled supplies since relatively few organisms would gain access to limited amounts of milk, and when this was mixed with other milk the dilution would be so great that the probable incidence of human infection would be relatively small. It might be argued that the contaminating streptococcus would multiply rapidly in the milk provided the temperature was favorable and that the product reaching the consumer would thus contain large numbers of the streptococci. But to explain severe outbreaks of scarlet fever through milk contamination one would have to assume that the organism gained access in goodly numbers and multiplied rapidly. However certain experiments here to be reported indicate that streptococci of the scarlet fever type are acted upon adversely by milk.

During the observations in connection with the artificial inoculation of Cow 1462 with a streptococcus of the human scarlet fever type a peculiar phenomenon was observed. It was customary to plate the milk from the involved quarters after logarithmic dilutions in salt solution, the first plate of a series containing a 10^{-1} dilution, the second a 10^{-2}, until to the final plate milk diluted 10^{-6} was added.

Toward the end of the observation, when the numbers of streptococci per cc. were not excessive, it was noted that the first plate culture contained streptococci in smaller numbers than one would expect from the number found in the higher dilutions. Furthermore the colonies were very small with correspondingly small hemolytic zones; yet when such colonies were subcultured and replated colonies of the usual size with well defined hemolytic zones developed. Two explanations suggested themselves, first, that the colonies were too numerous in the plate, and second, that an inhibitory substance had developed in the quarter which was active in the 10^{-1} dilution but not in the 10^{-2} dilution. Further observations indicated that overcrowding was not responsible for the change in the character of the colonies. The inhibition appeared to be a property of the milk and as such worthy of more careful study.

EXPERIMENTAL.

The cultures employed have been described in the preceding papers. They were carried for stock purposes on agar slants to which a few drops of defibrinated horse blood had been added. For the experiments transfers were made into veal infusion broth and incubated 16 hours. Inasmuch as the rest of the procedure varied, some details of the individual experiments are given separately.

Experiment 1.—Milk from individual quarters of Cow 1462 was drawn directly into sterile bottles and after chilling freed of fat by centrifuging. That from the quarters artificially infected with the hemolytic streptococcus was combined and portions of this milk and of that from an uninfected quarter were heated at 60°C. for 20 minutes, while other portions were not heated. In addition the cow was bled on the day before the experiment and the serum collected.

The inhibitory activities of the milk and serum were then tested in the following manner. Serum or milk was added in amounts of 0.5, 0.25, and 0.1 cc. to Petri dishes containing 0.5 cc. of defibrinated horse blood. A loop of dilute broth culture of the streptococcus obtained from Cow 1462 was added to 10 or 12 cc. of melted agar cooled to 48°C. and the whole plated. The plates were incubated for 48 hours at 38°C.

Inasmuch as the unheated milk contained many native organisms giving rise to many colonies in the plates, the results were imperfect and for this reason they will not be considered in detail, although it was clear that even under such conditions the growth of the streptococcus was inhibited.

The results of the first experiment are given in Table I.

The fact seems established by the experiment that when milk from either the infected or normal quarters was added to plate cultures containing small numbers of hemolytic streptococci a distinct inhibition resulted. The colonies were smaller and their hemolytic activity greatly diminished or even entirely suppressed, as when 0.5 cc. of milk from the two involved quarters was incorporated in the culture. In the series in which blood serum was mixed and incubated

TABLE I.

The Influence of Blood Serum and Milk on the Size of Colonies and Hemolytic Activities of the Streptococcus from Cow 1462.

	Plate containing	
	cc.	
Blood serum	0.5	30 colonies varying from 1.5 to 1.8 mm. Hemolytic zones 4 mm.
	0.25	20 colonies average 1.8 mm. Hemolytic zones 4 mm.
Milk heated 60°C., from uninfected quarter (R. F.)	0.5	No growth
	0.25	6 colonies averaging 0.4 mm. Indistinct hemolytic zones 0.8 mm.
	0.1	5 colonies 0.4 to 0.6 mm. Hemolytic zones 1.25 to 1.75 mm.
Milk heated 60°C., from involved quarters, R. H. and L. H.	0.5	No growth in 24 hrs. 48 hrs., 5 tiny, non-hemolytic colonies
	0.25	30 colonies 0.25 mm. Hemolytic zones 0.6 mm.
	0.1	6 colonies 0.025 mm. Hemolytic zones 0.75 mm.

with the culture material there was no inhibition. One may infer that the inhibiting property was not derived from the blood.

It seemed possible that the organism employed might be unique in sensitiveness to the inhibitory action of milk. Furthermore the incorporation of as much as 0.5 cc. of milk to the blood agar plate might perhaps alter the nutritive character of the medium and thus prevent normal growth. The more elaborate procedure employed in Experiment 2 was devised to control these features.

TABLE II.

The Effect of Milk on the Character of the Growth of the Scarlet Fever Streptococcus.

Culture	Milk	Plate containing	
		cc.	
F. C.	R. F., filtered	0.5	No growth 24 hrs. 48 hrs., 10 colonies, tiny, surrounded by faint hemolysis
		0.25	384 colonies, average 0.4 mm. Hemolytic zones 0.75 mm., indistinct
		0.1	345 colonies, average 0.6 mm. Hemolytic zones 1 to 1.25 mm., clear
	R. F., heated 58°C. 20 min.	0.5	320 colonies less than 0.25 mm. Hemolytic zones barely perceptible
		0.25	384 colonies average 0.25 mm. Hemolytic zones 0.5 mm.
		0.1	350 colonies, average 0.6 mm. Hemolytic zones 1 mm.
	R. H., filtered	0.5	325 colonies barely visible × 9. Non-hemolytic
		0.25	400 " " " × 9. "
		0.1	384 colonies, average 0.9 mm. Hemolytic zone 3 mm.
	R. H., boiled 5 min.	0.5	428 colonies, average 0.9 mm. Hemolytic zone 3 mm.
	None		384 colonies, average 0.8 mm. Hemolytic zone 3 mm.
Scarlet Fever 55	R. F., filtered	0.5	90 colonies visible × 9. Non-hemolytic
		0.25	576 colonies, less than 0.25 mm. Hemolytic zones 0.5 mm.
		0.1	580 colonies, average 0.5 mm. Hemolytic zones 2 mm.
	R. F., heated 58°C. 20 min.	0.5	No growth
		0.25	640 colonies less than 0.25 mm. Hazy hemolytic zones 0.5 mm.
		0.1	576 colonies 0.25 mm. Clear hemolytic zones 0.75 mm.
	R. H., filtered	0.5	No growth
		0.25	30 colonies visible × 9. Non-hemolytic
		0.1	179 colonies 0.25 to 0.3 mm. Clear hemolytic zones 0.6 mm.
	Boiled 5 min.	0.5	428 colonies 0.8 to 1 mm. Clear hemolytic zones 3 mm.
	None		284 colonies 0.8 mm. Clear hemolytic zones 3 to 3.5 mm.

Experiment 2.—Culture F. C. from the throat of a milk handler and Scarlet Fever Streptococcus 55 were employed in this experiment. Milk in separate bottles was obtained directly from the right fore and right hind quarters of Cow 1462. It was chilled and freed of fat. One portion of each was filtered through Berkefeld candle V, another lot heated at 58°C. for 20 minutes, and the remainder boiled for 5 minutes. The various lots in amounts of 0.5, 0.25, and 0.1 cc. were incorporated in the plate cultures. The effect on the growth of both strains is recorded in Table II.

The results of Experiment 2 resemble those of Experiment 1. 0.5 cc. of milk, filtered or heated at 58°C. for 20 minutes to rid it of native bacteria, proved sufficient to inhibit or entirely suppress the growth of the scarlet fever streptococcus. In certain instances although colonies of the organism developed in the Petri dishes no hemolysis occurred; nevertheless when such colonies were subcultured and replated in the blood agar mixture characteristic hemolytic colonies developed. The control to which 0.5 cc. of boiled milk was added failed to show appreciable inhibition.

Two series of the plate cultures were photographed. In order to afford a proper comparison they were magnified about 5 times. Figs. 1 to 5 are the photographs of the plate cultures of Strain F. C. without milk (Fig. 1), with boiled milk (Fig. 2), and the series in which 0.5, 0.25, and 0.1 cc. of filtered milk was added (Figs. 3, 4, 5). Figs. 6 to 10 show the effect of mixing milk heated at 58°C. for 20 minutes with the cultures of Scarlet Fever 55. Photographs of the two controls, one without milk and the other with boiled milk, are included for comparison.

It might be argued that the milk from Cow 1462 contained some immune property acquired as the result of partial recovery from infection. To test this possibility Experiment 3 was devised.

Experiment 3.—Milk was obtained directly from the udder of five normal cows chosen at random from those of a large herd. It was mixed and when freed of fat a portion was filtered, another heated at 58°C. for 20 minutes, and a third lot boiled for 5 minutes. It was then added in the usual amounts to the Petri dishes and its effect on cultures of Strain F. C. and Scarlet Fever V noted.

It was found after 48 hours incubation that the growth of Culture F. C. in the plates containing 0.5 cc. of either filtered milk or milk heated at 58°C. was completely inhibited. In plates containing 0.25 cc. either nearly complete inhibition occurred, or the colonies were too small to be seen with the unaided eye. Even

as little as 0.1 cc. of milk greatly diminished the size of the colony and the zone of hemolysis. The same could be said of Scarlet Fever V. In both series the addition of 0.5 cc. of boiled milk failed to diminish the number or size of the colonies although the hemolytic zones were a little smaller than in the control cultures which were made without milk.

It appears certain then that there is a natural inhibitory substance in milk which passes through a Berkefeld filter V and is not greatly injured when milk is heated at 58°C. for 20 minutes but inhibits the growth of streptococci of the scarlet fever type in plate cultures. The inhibition depends on the concentration of the milk but even when this is diluted as much as 1:100, as in the series in which 0.1 cc. of milk was mixed with 10 or 12 cc. of agar and 0.5 cc. of blood, its effect is readily visible.

Since cow's milk contains a principle which is inhibitory even when mixed with the culture medium, it seemed probable that exposure of the streptococci to the direct action of undiluted milk might result in definite destruction. To test this point milk was inoculated with the streptococcus and plated, in the manner outlined by Jones and Little (1), in the hope that the rate of growth or destruction could be measured. As might be expected from the preceding experiments, milk even when inoculated with 3,000 or 4,000 streptococci per cc., failed to show growth when plated in amounts of 1 cc. It was necessary to change the methods considerably before decisive experiments were obtained.

Experiment 4.—Milk from five cows chosen at random was mixed and freed of fat by centrifugation. A portion was heated at 58°C. for 20 minutes and the remainder boiled for 5 minutes. Both lots were then distributed into sterile agglutination tubes in amounts of 1 cc. The tubes were then separated into two groups each containing an equal number of tubes of the pasteurized and the boiled milk. Each tube of one group was inoculated with 1 loop of Strain F. C. diluted 500 times in broth. Those of the other group received a similar inoculation with Scarlet Fever V. Plate cultures were made by adding 0.25 cc. of milk to uniform amounts of blood and agar. Initial plates were poured and others after various intervals of incubation. The results of this experiment are recorded in Table III.

While there is some irregularity in the results recorded in Table III, the influence of the milk is evident. It is clear that fresh milk heated to 58°C. for 20 minutes actually prevents multiplication of

scarlet fever streptococci during incubation periods ranging from 2 to 48 hours. From the protocol it appears that the milk probably destroyed both strains of streptococci after an interval of 4 or 6 hours. To determine whether this was actually the case required further experimentation. It seemed possible that the organism subjected to the unfavorable influence of the milk heated at 58°C. was incapable of growth when plated because of the fact that the culture medium

TABLE III.

The Effect of Undiluted Milk on the Scarlet Fever Streptococcus.

Culture	Milk	Colonies developing in plate cultures containing 0.25 cc. milk						
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 24 hrs.	After 48 hrs.
F C.	Heated 58°C.	No growth	243 Visible × 12	256 Visible × 12	No growth	No growth	No growth	No growth. Reaction pH 6.6
F. C.	Boiled	409	4,708 Whole plate hemolyzed	72,000	Innumerable	Innumerable	Innumerable	Coagulates on boiling. pH 5.2
Scarlet Fever 55	Heated 58°C.	384	154 Visible × 12, non-hemolytic	No growth	No growth	No growth	No growth	No growth. pH 6.6
Scarlet Fever 55	Boiled	512	7,488	86,400	Innumerable	Innumerable	Innumerable	Coagulates on boiling. pH 5.4

contained 0.25 cc. of the inhibiting milk. This factor, added to the effect of previous exposure, might still be insufficient to kill the organism although suppressing its multiplication. That the same milk when boiled for 5 minutes was well adapted as a culture medium is obvious in the protocol, since multiplication was noted throughout the series.

In the next experiment only a trace of milk was added to the plate cultures.

Experiment 5.—Milk was obtained from the same cows as in Experiment 4. It was handled in a similar manner. After distribution in agglutination tubes in amounts of 1 cc., each tube was inoculated with a loop of broth culture diluted 500 times. Each tube before plating was centrifuged at high speed for 15 min-

TABLE IV.

Streptococci Surviving after Incubation in Milk.

Culture	Milk	Colonies developing in plate cultures						
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 24 hrs.	After 48 hrs.
F. C.	Heated 58°C. 20 min.	205	5	11	11	Sterile	Sterile pH 6.6	Sterile pH 6.6
F. C.	Boiled 5 min.	218	8,960	86,400	Innumerable	Innumerable	Innumerable pH 6.0 Thickens on boiling	pH 5.4 Coagulates on boiling
Scarlet Fever V	Heated 58°C. 20 min.	217	205	192	205	77	11 pH 6.6	Sterile pH 6.6
Scarlet Fever V	Boiled 5 min.	218	4,992	46,080	Innumerable	Innumerable	Innumerable pH 6.0	pH 5.6 Coagulates on boiling

TABLE V.

The Effect of Milk on Scarlet Fever Streptococci at 3° and 4°C.

	Before refrigeration	After refrigeration	
		24 hrs.	48 hrs.
Culture F. C. in milk heated 58°C. 20 min.	205	24	12
" " " boiled milk	218	300	412
" Scarlet Fever V in milk heated 58°C. 20 min.	217	156	102
" " " " " boiled milk	218	130	166

utes. The bulk of the milk was drawn off and 1 cc. sterile salt solution added and thoroughly mixed. The mixture was then withdrawn and added to the Petri dishes. In this way it was hoped that so little milk would be added to the medium that its effect would be negligible. The majority of the tubes were incubated

and plates prepared at indicated intervals. Some of the tubes were refrigerated at 3–4°C. and their contents plated by the same method after 24 and 48 hour intervals. The colonies were counted after an incubation of 48 hours in the blood agar medium. The results are given in Tables IV and V.

It is evident that the scarlet fever streptococcus fails to multiply in mixed milk provided the milk has not been heated sufficiently to destroy the inhibitory substance. It is also true that the principle in milk actually destroys the organism. The lethal effect is most marked at 38°C. At this temperature Culture F. C. was killed after 8 hours incubation. Scarlet Fever Streptococcus V was more resistant to the action of milk, since there was a more gradual diminution in the number of organisms, but after 48 hours none survived.

When the same cultures were exposed to the action of milk in the refrigerator Strain F. C. again proved more susceptible since only about 10 per cent of the streptococci survived for 24 hours, whereas culture Scarlet Fever V was not appreciably affected by the milk during a refrigeration of 24 hours or even 48 hours.

When the experiment was repeated at the temperature of the room, the number of streptococci was definitely diminished although not so greatly as at incubator temperature. The surviving streptococci failed to approach in number those implanted in milk and refrigerated.

DISCUSSION.

It can be said that milk heated at 58°C. for 20 minutes or filtered through the coarsest Berkefeld filter possesses the property of inhibiting the growth of the scarlet fever streptococcus. It also is true that the principle is sufficiently active to destroy certain of the organisms. That this phenomenon cannot be attributed to the lack of adaptation of the organism for growth in the food mixture represented by milk is amply shown by the behavior when boiled milk is used. It must be recognized that this activity is not specific for the streptococcus since it has been shown by others that other types of organisms are inhibited. However, it appears to be particularly potent for streptococci especially those of the scarlet fever type. The inhibitory and lethal effect of milk on the streptococcus is most marked at temperatures approaching that of the body. When artificially infected milk is stored at 3° or 4°C. more of the streptococci survive.

Since the evidence points to the udder as the source of origin of the inhibitory principle it is not surprising that the action of the latter should be most effective at a temperature about that of the organ in which it originates.

Milk added to plate cultures entirely prevents the development of colonies or so changes the appearance of the colonies as to make them unrecognizable. In many instances signs of hemolysis are not to be seen. Even when as little as 0.1 cc. of milk is added to the medium the surrounding hemolytic zone is so small that the colonies can easily be mistaken for the bovine type. These facts must be borne in mind when mixed milk is examined for human hemolytic streptococci.

Since the experimental evidence indicates strongly that milk, provided it is not heated at too high a temperature, will inhibit the growth or kill the scarlet fever streptococcus, the opinion that severe outbreaks of scarlet fever result from human contamination of milk must be viewed with considerable doubt. It might be objected that the experiments were artificial. But they were optimum conditions for the growth of the streptococcus and other organisms which would rapidly sour the milk under natural conditions were excluded. The change from a broth medium to one containing milk would appear to be no greater than that from a human throat to raw milk. That a few individuals may contract the disease through direct human contamination of milk is possible, but the occurrence of epidemics would imply a heavy inoculation of the milk. Infection of the udder of a single cow with the scarlet fever streptococcus and the resultant shedding of large numbers of the organisms into the milk,—phenomena recorded in our foregoing papers,—afford a more reasonable explanation of milk-borne epidemics.

SUMMARY.

The experiments indicate that milk filtered through a Berkefeld candle V or heated at 58°C. for 20 minutes when added to blood agar plate cultures interferes with the development of colonies of the scarlet fever streptococcus. The observed inhibition is proportional to the amount of milk. When the approximate milk dilution in the Petri dish is 1:20 or 1:25 growth of the organisms is completely suppressed or only a small proportion of non-hemolytic colonies develop.

As the amount of milk is decreased the colonies become larger and their hemolytic zones more pronounced, although even when the final dilution of milk reaches 1:100 or 1:125 only colonies easily mistaken for the narrow zoned bovine streptococci appear. The effects upon the surviving organisms would appear to be transient since both the non-hemolytic colonies and those with small zones manifest the original hemolytic properties when transferred to other media. When scarlet fever streptococci are added in small quantities to milk heated at 58°C. for 20 minutes and incubated growth is inhibited. If the period is prolonged the streptococci are killed. On refrigeration of such mixtures some of the streptoccci are killed but others survive the test period.

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EXPLANATION OF PLATE 38.

Magnification × about 5.

FIG. 1. Culture F. C. after 48 hours incubation in blood agar plate culture.

FIG. 2. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.5 cc. boiled milk.

FIG. 3. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.5 cc. filtered milk. There are no colonies of sufficient size to be detected at the magnification given.

FIG. 4. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.25 cc. filtered milk. Note the difference in size of colonies in this plate when compared with those in Figs. 1 and 2.

FIG. 5. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.1 cc. filtered milk. The colonies and hemolytic zones are larger than those in Fig. 4 but not as large as those in Figs. 1 and 2.

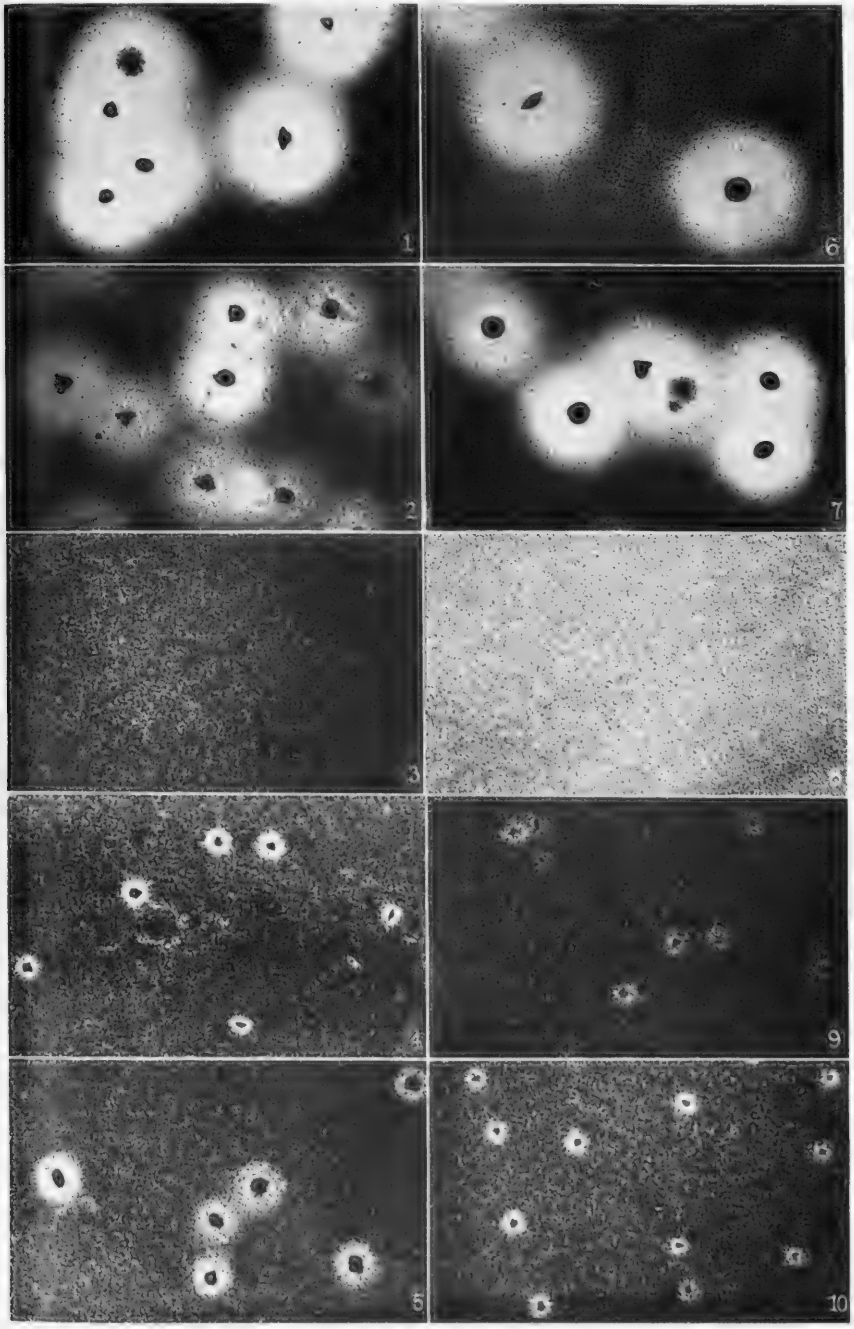
FIG. 6. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture.

FIG. 7. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.5 cc. boiled milk.

FIG. 8. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.5 cc. milk heated at 58°C. for 20 minutes. No growth visible at this magnification.

FIG. 9. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.25 cc. milk heated at 58°C. for 20 minutes. Note the small size of colonies and character of hemolytic zone as compared with Figs. 6 and 7.

FIG. 10. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.1 cc. milk heated at 58°C. for 20 minutes. The colonies and hemolytic zones are larger than those in Fig. 9 but not as large as those in Figs. 6 and 7.



Photographed by Louis Schmidt.

(Jones: Udder infection with streptococci. III.)

THE REGENERATION OF AUTOPLASTIC LYMPH NODE TRANSPLANTS.

By HENRY L. JAFFE, M.D., and MAURICE N. RICHTER, M.D.

(*From the Laboratory Division of the Hospital for Joint Diseases and the Pathological Laboratory of Bellevue Hospital, New York.*)

Plates 39 to 41.

(Received for publication, March 22, 1928.)

This work was undertaken as a companion study to that on transplantation of thymus previously reported (1). In spite of the extensive literature on tissue transplantation we found no references to the transplantation of lymph nodes. Since the results of these experiments were to be compared with those of thymus transplants in guinea pigs, we carried out the first experiment on this species. Our experiments were unsuccessful because the transplantation of cervical lymph nodes of stock guinea pigs invariably led to abscess formation, which, we believe, was due to the use of lymph nodes already infected. The necrotic tissue which results from transplantation is a favorable medium for the growth of bacteria present in the nodes. Even the use of young guinea pigs, about 25 days of age, frequently gave the same result. We abandoned the experiments on guinea pigs, and used albino rats, which are quite resistant to pyogenic infection. We used rats between 3 and 5 weeks of age, and transplanted cervical nodes which were normal in gross appearance. Successful transplants were obtained in over 90 per cent of the cases. Whole lymph nodes were planted in pockets of the abdominal wall, by the same technique that was used in the transplantation of thymus.

In all, 31 albino rats were used, in each of which two nodes were transplanted simultaneously. In this series of 62 transplantations there were but four instances of infection.

The transplants were studied at about 24 hour intervals from the 1st to the 12th days, and at longer intervals up to 35 days. Most of the plants were removed between the 2nd and 7th days, as this is the period of most active regeneration.

The tissues were fixed in Bouin's fluid, Helly's fluid, or alcohol. The Bouin- and some of the Helly-fixed tissues were embedded in paraffin and stained with hematoxylin and eosin, Van Gieson, iron-hematoxylin, or phosphotungstic acid-hematoxylin. The plants fixed in alcohol were stained with pyronine and methyl green. Some of the Helly-fixed nodes were sectioned in celloidin, and stained with azure B-methylene blue-eosin (2). This last method gave the best preparations for studying the details of regeneration.

OBSERVATIONS.

Minor differences were observed in transplants of the same age even in the same animal. The following account is an approximation of the changes observed with reference to the time after transplantation.

At the end of 24 hours, there is extensive necrosis in the center of the transplant involving mainly the lymphocytes. At the periphery of the node some normal appearing lymphocytes are seen. Surrounding the node is a space, probably the peripheral lymph sinus of the transplanted node, which in some areas contains a few lymphocytes. Most of the reticulum of the transplant is preserved, though obscured in the central portions by the cellular debris. The vessels in the tissue around the transplant are not dilated, and the only reaction to the transplant is a slight polymorphonuclear infiltration between the muscle fibers about the transplant.

By the end of the 2nd day, three well defined areas are visible in the transplant: a central necrotic area, a peripheral margin of well preserved tissue, and an intermediate zone of hyperplastic reticulum (Figs. 1 and 2). Considerable liquefaction and absorption have taken place, so that the necrotic area is reduced in size. The reticulum contains numerous mitotic figures. The marginal zone contains mainly small lymphocytes and an occasional large lymphocyte. Mitotic figures in the lymphocytes are difficult to find. At the hilus of the node dilated vessels filled with blood penetrate the transplant, and their subdivisions extend to the periphery. The cellular reaction about the transplant is not extensive.

During the 3rd day, the necrotic mass becomes smaller, and the reticulum becomes more prominent. Large phagocytic cells in the reticulum aid in the removal of the debris. At the margin is a zone of small lymphocytes, among which are a few large lymphoid cells. Large lymphoid cells are formed from the reticulum in areas where reticulum cell hyperplasia is most prominent, and are therefore formed extravascularly. The formation of lymphocytes from tissue cells is also observed in the connective tissue outside the transplanted node.

During the 4th day, the debris is almost completely removed. The transplant now appears more like a normal lymph node, with very vascular connective tissue at the hilus (Fig. 3). Extravascular formation of lymphocytes from tissue cells is observed in areas both in and outside of the node. The peripheral lymphocytic

zone is wider. There are no germinal centers, but scattered small groups of large lymphoid cells, identical with germinal center cells, are observed. There is a tendency for the lymphocytes to be arranged in cords, extending from the periphery to the medullary portion.

After 5 days, regeneration is nearly complete. The transplant now appears as a normal lymph node. The lymph capillaries at the hilus are filled with lymphocytes and are efferent vessels (Fig. 4). The reticulum is still prominent, and mitoses may be observed. Large lymphocytes are scattered among the smaller cells of both the cortex and medulla.

At 6 days the node is completely regenerated. The efferent capillary lymph vessels previously observed may be traced from the hilus into the surrounding tissue.

At periods from 6 to 35 days, sections show that the transplant persists as a fully developed lymph node in the muscles of the abdominal wall. No retrograde changes were noted (Figs. 5 and 6).

DISCUSSION AND SUMMARY.

The reticulum plays an important part in the regeneration of lymph nodes autoplastically transplanted into the abdominal wall of the albino rat. The necrosis which follows transplantation involves mainly the lymphocytes. A margin of lymphocytes is preserved only at the periphery. This may be due to early lymphatic connection with the marginal sinus of the node. The reticulum cells are apparently more resistant since for the most part they do not degenerate. There are three possible origins of the lymphocytes of the regenerated node. They may arise by proliferation of lymphocytes retained at the periphery of the plant. The presence of a few mitotic figures among lymphocytes in the marginal region confirms the possibility of this mode of origin. They may also be derived from lymphocytes brought into the transplant by the blood or lymph circulation. Though some lymphocytes are present in the marginal sinus, and an occasional lymphocyte is seen in the capillaries which enter at the hilus, we believe that this source of origin of the lymphocytes is negligible. On the other hand, the hyperplastic reticulum appears to be the important source of lymphocyte production. These may be derived from the reticulum directly as small lymphocytes, or may be formed through the intermediary stage of large lymphoid cells. This capacity is not limited to lymphatic reticulum, as small foci of lymphocyte formation are found in the connective tissue in the vicinity

of the transplants. The reticular origin of lymphocytes is most easily observed in the earlier stages of regeneration before the picture is obscured by the numerous small lymphocytes.

Two structures in the regenerating node are directly traceable to the same structures of the transplant. These are the marginal sinus and the hilus. The marginal sinus of the transplant is preserved, and is probably an important means by which lymphatic communication is established with the surrounding tissue. The hilus is the site of entry of the blood vessels, and the hilus of the regenerated node is the same as that of the transplant.

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EXPLANATION OF PLATES.

PLATE 39.

FIG. 1. 2 days. Shows three well defined areas. Central necrotic zone, zone of well preserved lymphocytes, and intermediate zone of hyperplastic reticulum. Hematoxylin and eosin. ×37.

FIG. 2. Higher magnification of the three zones shown in Fig. 1. Hematoxylin and eosin. ×112.

PLATE 40.

FIG. 3. 3 days, 17 hours. The cellular debris has practically disappeared and its place is taken by a vascular reticulum with many newly formed capillaries. There is a peripheral zone of normal appearing lymphocytes. Eosin-azure B-methylene blue. ×72.

FIG. 4. 5 days. Almost complete regeneration, but with prominent reticulum in the center. Efferent lymph channels contain many lymphocytes. Hematoxylin and eosin. ×55.

PLATE 41.

FIG. 5. 7 days. Complete regeneration of transplant. Hematoxylin and eosin. ×32.

FIG. 6. 21 days. Regenerated lymph node transplant after 21 days. No retrograde changes. Hematoxylin and eosin. ×37.

FIG. 1.

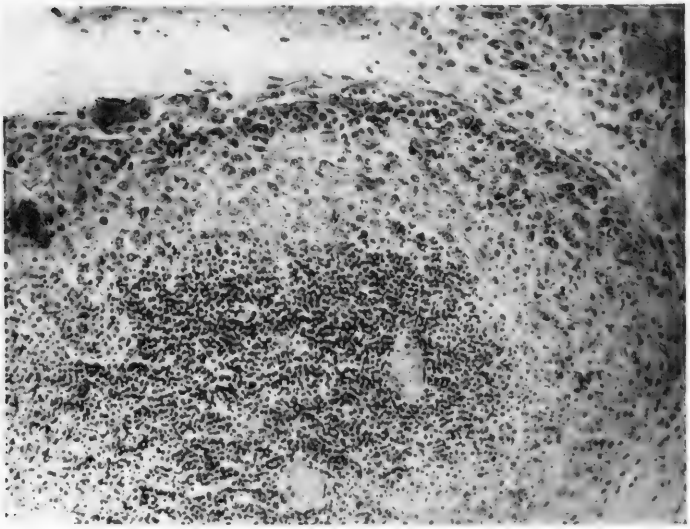

FIG. 2.

(Jaffe and Richter: Autoplastic lymph node transplants.)

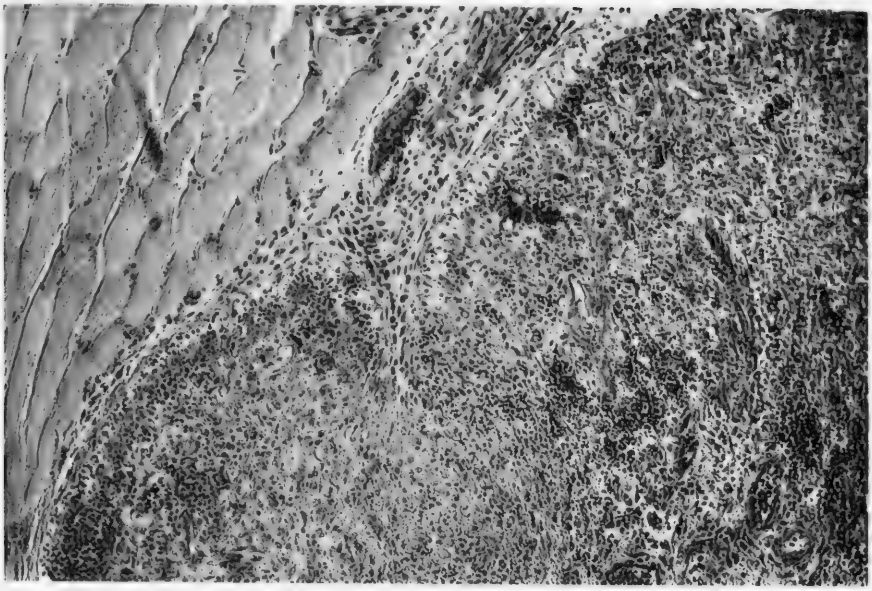

FIG. 3.

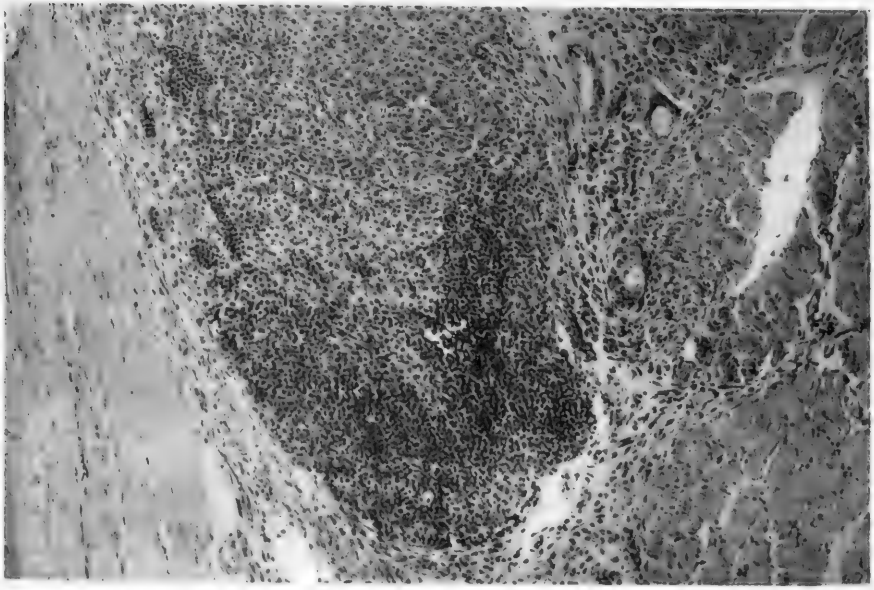

FIG. 4.

(Jaffe and Richter: Autoplastic lymph node transplants.)

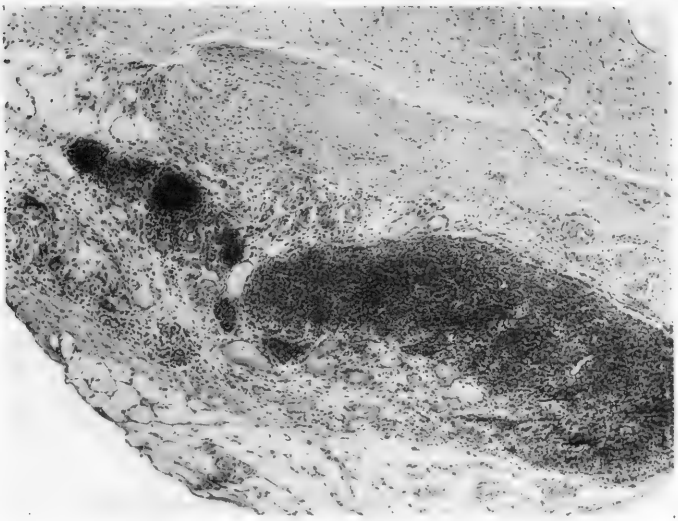

FIG. 5.

FIG. 6.

(Jaffe and Richter: Autoplastic lymph node transplants.)

UNIV

THE AUTOPLASTIC TRANSPLANTATION OF TISSUES INTO THE BONE MARROW CAVITY.

I. The Thymus.

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Plates 42 to 44.

(Received for publication, March 22, 1928.)

The relation of lymphoid tissue to bone marrow is not definitely known. According to the adherents of the dualistic theory of blood formation these two tissues are independent or even antagonistic to each other. Thus the possibility of a lymphocyte differentiating into a myeloid cell is denied.

The unitarian theory of blood cell development postulates a close relationship between the lymphoid and myeloid tissues, and lymphocytes are considered capable of differentiating, directly or indirectly, into myeloid cells. Maximow (1), Downey (2), and others have reported such findings. Downey has recently published a comprehensive review on this subject. Maximow (3) induced this change experimentally by the addition of cell-free bone marrow extracts to lymphoid tissue cultures.

We transplanted tissues into the bone marrow cavity, in direct contact with actively growing marrow, to study the effects of the marrow on the transplanted tissues, and of the tissues on the bone marrow. Three tissues of lymphoid nature—lymph node, spleen, and thymus—were transplanted. It was thought that if these tissues were genetically and biologically different, different reactions might be produced both in them and in the bone marrow on transplantation.

The lymph nodes are composed almost entirely of lymphocytes and their supporting stroma. Cytologically the spleen differs from lymph nodes, in that a small amount of myeloid tissue may be present, though overshadowed by the lymphocytes. The lymphoid nature of the

thymus is questionable because opinion is not unanimous concerning the "lymphoid" nature of the cortical cells. By the introduction of these tissues into the marrow cavity similarities and differences in their reactions were observed. This paper deals with transplantation of the thymus.

Maximow (4), Danchakoff (5), Hammar (6), and others regard the small thymic cells as lymphocytes. Stöhr (7), Jaffe (8), and others regard them as epithelial elements. In experiments on myeloid metaplasia Danchakoff reported the transformation of thymic cortical cells into granular myelocytes, a change supposedly characteristic of lymphocytes and their precursors.

The literature on transplantation of organs into the bone marrow is scant, and we have found no references to transplants of thymus, lymph nodes, or spleen in this situation.

Methods.

Young guinea pigs 8 to 11 weeks of age were used. These animals were reared in the laboratory under hygienic conditions, and were apparently healthy. Under ether anesthesia with aseptic precautions both lobes of the thymus were removed, and placed immediately in sterile physiological salt solution kept at about 38°C. An osteotomy was then performed on the upper third of each tibia. The marrow cavity of the upper third of the bone was curetted and wiped out with gauze. The thymus was then introduced into the cavity. Thirteen animals, in which the wounds healed by primary union, were used for this study. In six animals several fragments of thymus not more than 2 mm. in diameter were introduced into each marrow cavity, and in seven cases one large piece of thymus sufficient to fill the entire defect was inserted. The bone flap was replaced whenever possible, and the wounds were closed with fine silk.

The animals were killed at intervals of from 1 to 10 weeks after operation. The tibia was sawed through with a fine jeweler's saw at several levels, fixed in Helly's fluid, decalcified in 5 per cent nitric acid, sectioned in paraffin, and stained with hematoxylin and eosin.

The results of the transplantation of several small pieces were essentially the same as those which followed the transplantation of one large piece. Therefore both series of experiments are described together.

OBSERVATIONS.

At the end of 1 week, the transplanted thymus occupied the center of the marrow cavity and showed extensive degeneration of the small thymic cells and the Hassall's corpuscles, the thymic reticulum remaining for the most part intact. At several places in the peripheral portion of the transplant the thymic reticulum was hyperplastic and contained mitotic figures. A layer of loose cellular and vascular granulation tissue, in which new bone was forming, separated the thymus from the bone cortex. (Fig. 1.)

By the 2nd week much of the debris had been cleared away mainly by the process of liquefaction. Phagocytes were present in remarkably small numbers. At this time the outstanding feature in the transplant was the proliferating reticulum, with some round cells and an occasional polymorphonuclear leucocyte scattered in its meshes. During the 2nd week the small thymic cells reappeared and increased in number. The reticulum formed cell masses, the centers of which degenerated resulting in typical Hassall's corpuscles. We found no evidence of Hassall body formation from capillary endothelium as recently described by Jordan (9). In this stage new bone trabeculæ were forming around the transplant. (Fig. 2.)

During the 3rd week the thymus was usually completely regenerated, forming large cellular lobules, some of which showed differentiation into cortex and medulla. The thymic lobules were richly cellular and the small round thymic cells predominated. The Hassall's corpuscles were well developed. The regeneration of the thymus was complete in that all the structures of the normal thymus in their normal morphological relationship were reproduced. The regeneration, however, fell short of reproducing as active and cellular a thymus as was introduced. This is in marked contrast to the results obtained when thymus is autoplastically transplanted into the abdominal wall (8). The bony trabeculæ observed in the previous stage formed an incomplete bony capsule around the thymus. Where the bony wall was lacking, a thin layer of fibrous tissue separated the thymus from the marrow. (Figs. 3 and 4.)

By 6 weeks the bony capsule was fully developed and completely enclosed the thymus. The development of this bony capsule not

only prevented further growth of the thymus, but led to its atrophy. As the gland atrophied there was replacement fibrosis and Hassall's corpuscles disappeared. (Fig. 5.)

By $8\frac{1}{2}$ weeks only a tiny fragment of thymus remained which was separated from the marrow cavity by a capsule of newly formed bone (Fig. 6). The thymus was markedly atrophic, the cortical cells had almost completely disappeared, and practically nothing remained but a stroma consisting mainly of connective tissue, in the center of which was a small group of round cells. Reticulum cells, if present, could not be differentiated from the cells of the fibroblastic stroma. No Hassall's corpuscles were seen. A few foreign body giant cells were present in the stroma.

In sections made at the end of 10 weeks, no thymic tissue was found.

SUMMARY.

When the thymus is autoplastically transplanted into the marrow cavity of the tibia of a guinea pig, the transplant degenerates, regenerates, and finally atrophies. The degeneration and regeneration of the thymus when so transplanted are similar to the homologous changes occurring when it is autoplastically transplanted into the abdominal wall. For comparative study the material used in previously published work on transplantation into the abdominal wall was reviewed. A difference in the final result of transplantation of thymus in these two situations is observed. In the abdominal wall the thymus regenerates and persists apparently indefinitely. Abdominal wall thymus transplants more than a year old have been observed. In the bone marrow cavity, the regeneration, which is slower and less abundant, is followed by an atrophy of the gland, and finally its complete disappearance. This atrophy is, we believe, the result of the encasement of the thymus in a bony capsule. That the genetic character of the transplanted tissue has much to do with the success of the transplantation in the marrow, is evident from the fact that some other tissues regenerate without encapsulation, even though they may finally disappear.

The blood clot about the thymus which fills the rest of the curetted cavity, is quickly vascularized and replaced by regenerated cellular bone marrow and newly formed bony trabeculæ.

The difference in the fate of the thymus when transplanted in the abdominal wall and in the marrow, is probably due to differences in the reciprocal relations of the thymus to the tissues in those situations. From experience gained in the transplantation of other tissues into the marrow cavity, we have learned that encapsulation of the transplant does not occur with all tissues. The reaction of the marrow to transplanted thymus is similar to its reaction to any tissue foreign to it.

CONCLUSIONS.

1. The thymus when transplanted autoplastically into the bone marrow undergoes complete but not abundant regeneration.

2. The histological structure of the regenerated thymus is the same as that of the normal thymus despite its situation in the marrow cavity.

3. We have noted no metaplasia of the thymic round cells into myelocytes.

4. The regeneration of the bone marrow in the curetted cavity is not influenced by the presence of the thymus.

5. The regeneration of the thymus is accompanied by its enclosure in a fibrous and bony capsule which prevents extensive development and causes pressure atrophy of the transplant.

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6. Hammar, J. A., The new views as to the morphology of the thymus gland and their bearing on the problem of the function of the thymus, *Endocrinology,* 1921, v, 543, 731.
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Jaffe, H. L., Autoplastic thymus transplants. II. With particular reference to the regeneration of the reticulum cells and the formation of Hassall's corpuscles, *J. Exp. Med.*, 1926, xliv, 523.

9. Jordan, H. E., and Horsley, G. W., The significance of the concentric corpuscles of Hassall, *Anat. Rec.*, 1927, xxxv, 279.

EXPLANATION OF PLATES.

PLATE 42.

FIG. 1. 7 days. Cross-section of the tibia at the level of the transplant showing several lobules of degenerated thymus. The section also shows the replaced bone flap. Hematoxylin and eosin. ×18.

FIG. 2. 14 days. Regenerating thymus is surrounded by newly formed endosteal trabeculæ. Hematoxylin and eosin. ×18.

PLATE 43.

FIG. 3. 24 days. The regeneration of the thymus has reached its maximum. Hassall's corpuscles and medullary centers are seen. Marrow has regenerated between the trabeculæ, and the bony encapsulation is progressing. Hematoxylin and eosin. ×44.

FIG. 4. Higher magnification of the regenerated thymus shown in Fig. 3. Typical Hassall's corpuscles are seen. Hematoxylin and eosin. ×100.

PLATE 44.

FIG. 5. 41 days. The encapsulation of the thymus has excluded it from the marrow cavity. The organ lies between the osteotomy flap and the newly formed endosteal trabeculæ which separate it from the regenerated marrow. Retrograde atrophy of the thymus has set in. Hematoxylin and eosin. ×22.

FIG. 6. 63 days. The thymus is completely surrounded by a bony capsule connected with the cortex. There is almost complete atrophy of the thymus. Hematoxylin and eosin. ×25.

FIG. 1.

FIG. 2.

(Richter and Jaffe: Transplantation into bone marrow cavity. I.)

FIG. 3.

FIG. 4.

(Richter and Jaffe: Transplantation into bone marrow cavity. I.)

FIG. 5.

FIG. 6.

(Richter and Jaffe: Transplantation into bone marrow cavity. I.)

UNIV. OF MICH.

SURFACE TENSION OF SERUM OF THE SENSITIZED GUINEA PIG.

I. Surface Tension Changes Incident to the Process of Sensitization.

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(Received for publication, March 5, 1928.)

In a study of surface tension in the immunized rabbit, du Noüy[1] found a certain definite progressive change to take place, but concluded that this change is "not directly correlated to the presence of antibodies, but that the two phenomena are simply co-existent at the beginning of immunization."[2]

This conclusion suggested a study of surface tension changes in the serum of the guinea pig incident to the process of sensitization, since the early stage in immunization in the rabbit—when tissue changes take place and when the protective mechanism of circulating antibodies is not yet in operation—is comparable to the stage generally accepted as prevailing as a permanent state in the guinea pig. Zunz and la Barre,[3] using the drop method of measurement, did not observe any change in surface values as a result of serum sensitization. But it is to be noted that an examination was not made until after a period of 21 days following the sensitizing dose.

[1] du Noüy, P. L., *J. Exp. Med.*, 1925, xli, 779.

[2] du Noüy, P. L., Surface equilibria of biological and organic colloids, Chemical Catalog Company, New York, 1926, 120.

This volume presents the collected studies of du Noüy to which reference is made in this paper. The original papers appeared in the *J. Exp. Med.*, 1922, xxxv, 575, 707; xxxvi, 115, 547; 1923, xxxviii, 87, 659; 1924, xl, 129; 1925, xli, 663, 779; xlii, 9.

[3] Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1925, xcii, 223.

Technic.—The apparatus and technic as described by du Noüy were generally employed, with the following variations:[4]

1. An extension of the table carrying the watch-glass was made by superimposing a revolving table of wood large enough to carry five glasses.

2. Distilled water with a surface tension measurement of 73.0 dynes / cm. or more was accepted as meeting the requirements of a technic where only comparable values were of importance.

3. Careful checking of saline and glassware was carried out at the beginning of each test by reading a sample of saline which had been passed through all glassware. In addition, a sample of saline was read in each watch-glass.

4. Samples of sera which did not give an initial reading within 1 dyne of that of water were discarded.

5. In the second part of the study, dilutions of whole blood were used instead of serum or plasma. Only comparative results were required in the study, and whatever error the presence of blood cells might occasion was considered a constant factor.

EXPERIMENTS.

The work of du Noüy was repeated with three rabbits in the process of anti-horse serum precipitin production and with two untreated rabbits. He found that the static surface tension value of the serum of the treated rabbits was appreciably lowered. (This difference in the initial and the 2 hour, or static, readings he refers to as the *time-drop.*) The only noted variation was in the high values of the time-drop for these apparently normal animals. But at the end of the experiment, one, a discard from certain staphylococcus experiments, was found to have multiple kidney abscesses, and the other coccidiosis hepatis.

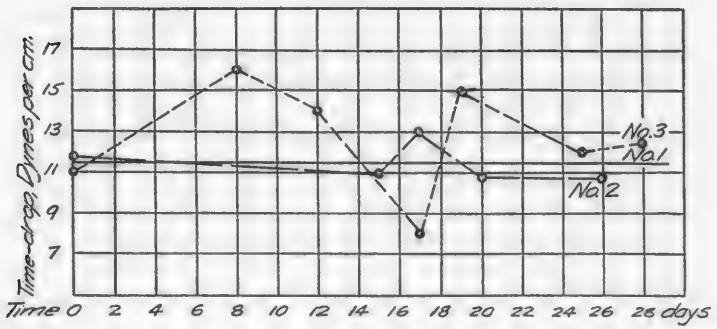
In the experiments on guinea pigs, a series of eight animals of about 200 gm. was sensitized with 0.1 cc. horse serum, intraperitoneal; another series of five was sensitized with 0.05 cc. horse serum, intravenous.

The critical concentration for giving the maximum time-drop values was found to be near that for the rabbit, 1/10,000, as found by du Noüy. This dilution was that used uniformly throughout the first part of this study, and the time-drop was for a 2 hour period.

[4] An adequate criticism of the ring method of measurement of surface tension and a discussion of the difficulties of interpretation of the resulting data have recently appeared by Johlin, J. M., *J. Gen. Physiol.*, 1928, xi, 301.

An average of 46 readings of normal guinea pig sera, including the first readings of the test animals, gave a time-drop value of 11.5 dynes. When a series of four animals was carried through a period of 25 days, the variation from this average was not found to be more than 2 dynes in any given animal, with a maximum and minimum value difference of 4.2 dynes.

The series treated with 0.1 cc. horse serum showed a maximum increase of 7.6 dynes on the 7th day. (More frequent bleedings were avoided because of the possible effects of an induced anemia.) About the 16th day there was a fall below the normal level followed by a rise.



TEXT-FIG. 1. Surface tension changes in guinea pig serum:

1. Average time-drop of normal serum, 46 readings.
2. Variations in time-drop in a series of four normal animals carried through the same period as 3.
3. Variations in the time-drop in a series of seven sensitized animals.

For this fluctuation no explanation is offered. It is also to be noted in du Noüy's findings for rabbit immune serum.[5] After the 25th day, the time-drop fell toward the normal level and remained at that point indefinitely—the average time-drop reading for this group being 12 dynes on the 45th day and 11.8 dynes on the 60th day after the sensitizing treatment. These changes are represented in Text-fig. 1.

In certain animals, the time-drop reached a value of 19 or more dynes, an increase of nearly 100 per cent of the initial reading.

[5] du Noüy, P. L., *J. Exp. Med.*, 1923, xxxvii, 665.

The experiment was repeated with another series of five animals sensitized with 0.05 cc. horse serum, intravenous. A like result was obtained, although the variations from the normal were not so great. This may have been due to the following factors: the initial readings for the series were above normal, suggesting the effect of an obscure infection; the series was smaller; the sensitizing dose was smaller than in the first series, thereby reducing the toxic effect of the serum.

That the effects noted might be charged simply to changes resulting from the toxicity of a foreign serum for particular or various tissues was suggested and the following experiment was carried out: Four of the animals used in the group of normal controls were given a solution of 1 mg. of uranium nitrate subcutaneously. Three of the normal group were kept for controls.

Evidences of a severe infection appeared in the whole group by the 4th day, all the animals losing from 17 to 70 gm. in weight. The animals which had had uranium nitrate suffered the greatest loss of weight, appeared sicker, and two were found dead on the 8th day. The curve for the time-drop rose on the 8th day, but for both treated and untreated animals alike, as in the serum-treated series. This confirms du Noüy's findings for the effect of an epidemic—snuffles, for example, in rabbits. At any rate, the surface tension changes following an acute infection could not be said to be augmented by an added toxic agency.

Du Noüy showed in his study of immune serum in the rabbit that no further response in surface tension behavior could be elicited by a repetition of the treatment which had called out the first change. The question was suggested as to whether or not a treatment with a different antigen might occasion a comparable rise in the time-drop. Three of the series used in the earlier experiment to show the changes following horse serum treatment were give 0.05 cc. egg white, intraperitoneal. Two others were given uranium nitrate, as described. In no case was there an increase in the time-drop. One of the uranium nitrate animals died on the 8th day. All the others gained weight and remained normal in appearance.

CONCLUSIONS.

The change in surface tension behavior in the serum of sensitized guinea pigs is, as du Noüy has concluded for immunized rabbit serum, not referable to an antibody content, since we know that the capacity for transfer of sensitization remains in the serum indefinitely, while the increased time-drop phenomenon is a transitory manifestation. That this phenomenon cannot be invoked by a new antigen capable of calling out its specific antibody would seem to make this response one due to some basic stable alteration of a tissue active in the general process of sensitization. That this alteration is not one called out by such a simple toxic injury as a uranium nitrate nephritis is contributory evidence that the primary toxicity of the horse serum is not the specific factor involved.

SURFACE TENSION OF SERUM OF THE SENSITIZED GUINEA PIG.

II. Surface Tension Changes in the Blood in Anaphylactic Shock.

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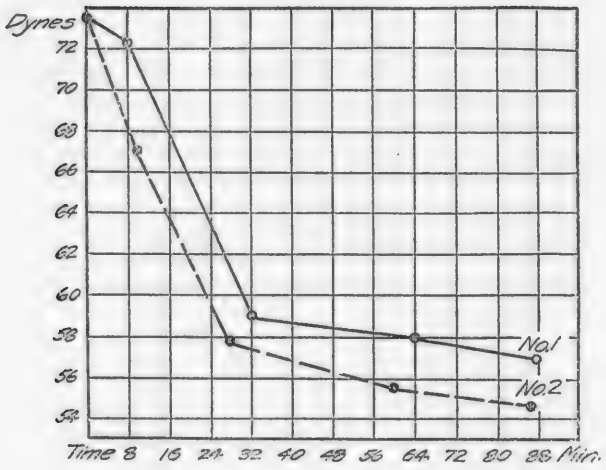
Certain workers in Belgium and France have studied the surface tension of guinea pig serum in sensitization and shock, using a modification of the drop method.[1–7] They found, in general, that there is a lowering of the dynamic value of the surface tension whenever the clinical symptoms of shock are manifest.

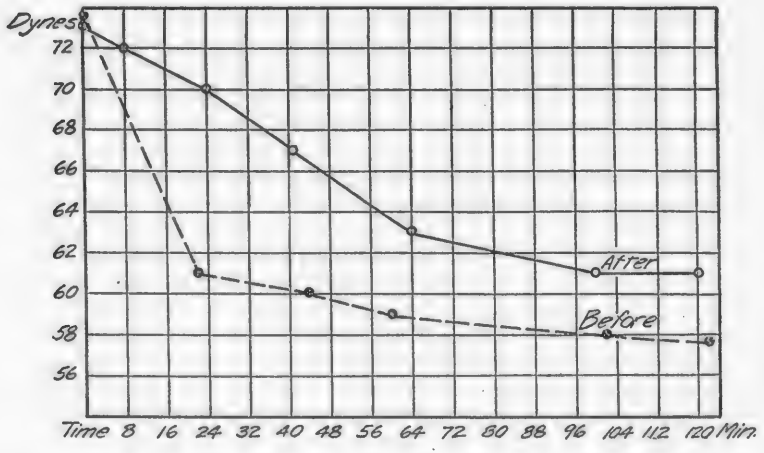
In a tentative trial to corroborate these findings, using a technic measuring the static value of surface tension in dilutions of serum of guinea pigs in anaphylactic shock, we could not find appreciable variations from the normal or preshock values.

This failure suggested the use of whole blood for a comparative study by which the delay in measurement, and the influence therefrom of any progressive changes inaugurated in shock, might be avoided. This was found to be a practical procedure if the blood was transferred directly from the syringe to an amount of saline sufficient to inhibit clotting. In addition to static values, such preparation might be expected to show any inherent differences in the rapidity of a change in surface tension values, thereby giving different types of curves as a function of time.

[1] Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1925, xcii, 223.
[2] Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1924, xc, 118, 659.
[3] Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1926, xcv, 858.
[4] Zunz, E., *Bull. Acad. roy. méd. Belgique*, 1925, v, 334.
[5] Kopaczewski, W., *Compt. rend. Soc. biol.*, 1919, lxxxii, 590.
[6] Kopaczewski, W., *Arch. phys. biol.*, 1922, ii, 1.
[7] Zunz, E., and la Barre, *Arch. internat. physiol.*, 1923, xxi, 361.

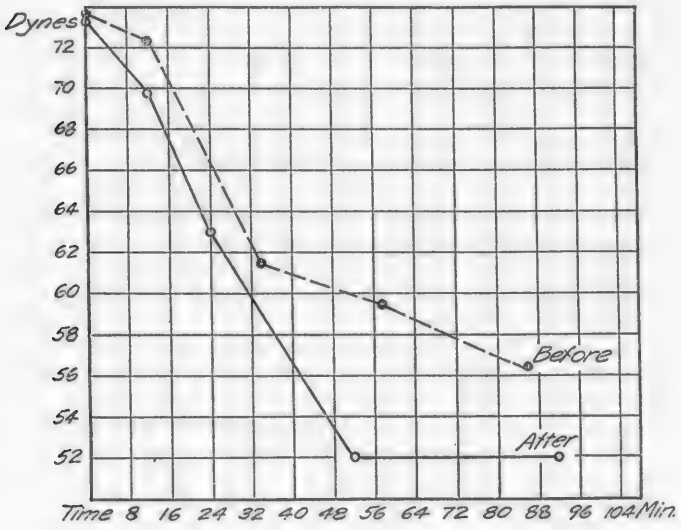


TEXT-FIG. 1. Surface tension readings of normal guinea pig serum.
1. Before an injection of horse serum, 0.5 cc.
2. About 1 minute after.



TEXT-FIG. 2. Surface tension readings before and after immediate fatal anaphylactic shock.

A preliminary study was made on the serum of an immunized rabbit. The critical concentration for the maximum 2 hour time-drop value was found to lie around 1/5,000. But it was found that such a whole blood dilution gave a static value reading lower than a serum dilution of 1/10,000 by about 5 dynes, and that the solutions gave more regular values; the time-drop in the same rabbit's serum on 3 different days showing less than a dyne variation. Following an attempt at shock in the same animal, where the only sign of a dis-



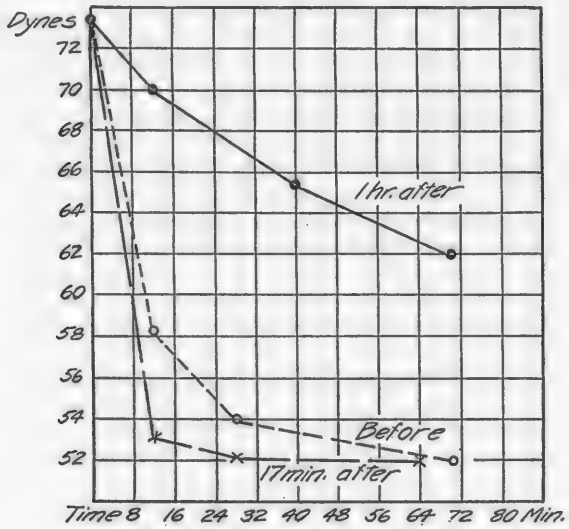
TEXT-FIG. 3. Surface tension readings before and after fatal, but not immediate, shock.

turbance was a heavy drowsiness, the static value reading was lowered by 3 dynes.

When a careful, repeated titration for a critical concentration for normal guinea pig whole blood was made, the lowest static values were found to occur around 1/8,000 with a second low value around a dilution just greater than 1/10,000. The former concentration was chosen for the ensuing study. That this does not represent serum alone is shown by the difference in static surface tension values for

the same dilution—lower by 7 dynes for blood than serum alone. A similar change in the critical concentration was noted by du Noüy for rabbit plasma.[8]

In preparation for these experiments a special collection of horse serum was made with all the precautions as to glassware that were taken in the general work. Eleven animals remaining from the study of sensitized serum, with a sensitization period of 60 to 90 days, and



TEXT-FIG. 4. Effect of cholesterol, 0.5 to 1.0 mg., intravenous, upon surface tension readings.

two others with a 10 day period, together with twelve normal animals were used for the experiment.

The effect of an intravenous injection of horse serum into normal guinea pigs gave a fall in the time-drop of about 2 dynes. Comparative curves for such a normal are shown in Text-fig. 1.

In case of shock, there was a considerable variation in values—from a rise in one case of a 5 dyne time-drop, to a fall of like value. Where

[8] du Noüy, P. L., *J. Exp. Med.*, 1927, xlvi, 1.

there is variation in the factors of (1) size of dose, (2) of time-interval between injection of serum and withdrawal of test blood, (3) of time-interval between injection of serum and onset of shock symptoms, such irregular findings are not surprising. In general, it can be shown that the first response to a dose of homologous antigen is a rapid fall in surface tension, and that this is greater in direct proportion to the degree of reaction as shown in the usual anaphylactic manifestations, or to the time elapsing between the injection of the serum and the withdrawal of blood.

In cases where the sensitized animal failed to show signs of shock, or where death occurred immediately after this dosage (less than 1 minute), or where death was delayed for several minutes, the curves representing the surface tension readings before and after the serum injection were not markedly different from those shown by the normal animal after such injection; but in certain cases the curves for the postinjection blood followed a higher level. In these, the differences in the curves tended to disappear after about 60 minutes, but the relative positions of the levels did not change (Text-fig. 2). When death is delayed for more than 1 minute and blood is taken after full development of shock symptoms, a definite lowering occurs in the resulting curves (Text-fig. 3).

The divergent variations in findings are not surprising when one reflects upon the very complex mixtures with which one deals and upon the antagonistic behavior of such colloidal solutions—this influenced, in turn, by relative concentrations, as shown by du Noüy for serum solutions and sodium oleate. Such active substances as cholesterol and lipoids have been shown to be increased in the blood following shock.[9,10]

This was verified as to an increase amounting to 16 per cent in cholesterol content of whole blood following shock in the guinea pig. A corresponding active effect upon the blood of a normal guinea pig was shown (Text-fig. 4) following the intravenous injection of a suspension of 1 mg. of cholesterol. *In vitro* a fall was followed by a rise when the cholesterol was used in a dilution of 1/20,000. When

[9] Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1925, xciii, 1044.
[10] Van de Velde, J., *Compt. rend. Soc. biol.*, 1927, xcvii, 1825.

powdered cholesterol was added directly to the serum dilution, a fall of 11 dynes occurred at once, and recovery did not take place. The surface tension of a mixture of saline and powdered cholesterol was lowered by 22 dynes.

In a few experiments with histamine shock, the histamine alone did not lower the surface tension of saline, but did lower that of a serum solution *in vitro*. When the blood was examined after shock, the same serum might exhibit a rise in surface tension following a sublethal dose, and a return to normal at a later period, or a marked fall following a dose sufficient to bring on an immediate and fatal shock. We are here probably concerned with the behavior of serum plus histamine, and each of these or their reaction product with liberated tissue substances (glycogen, cholesterol, etc.), where the effect may be primarily additive and secondarily antagonistic.

CONCLUSION.

The primary change in surface tension of serum incident to anaphylactic shock is probably due to a lowering of the surface tension of the serum by the addition of the antigen serum. But this may be followed by a further decrease or by an increase depending on the intensity and duration of certain secondary tissue changes.

THE PATHOGENESIS OF EARLY OBSTRUCTIVE JAUNDICE.

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INTRODUCTION.

For many years investigators have been concerned with the question of the mechanism of early obstructive jaundice, some studying the process as a whole, others limiting themselves to certain aspects of the problem.

In cases of complete obstructive jaundice of long standing, the mechanism is fairly well understood. As shown by Eppinger (1) for the first time, and confirmed by Abramow and Samoilowicz (2), the bile canaliculi in these cases are distended and tortuous and their walls frequently ruptured, the bile emptying into the pericapillary lymph spaces. These observations lead to the theory of increased tension of the bile capillaries due to bile continually flowing from the liver cells but unable to reach the duodenum. As a result of increased tension the vessel walls rupture and the extravasated bile is absorbed either by the blood capillaries or by the lymphatics. It then finds its way into the blood stream and is partly excreted in the urine and partly deposited in the tissues. This theory however, especially in the initial stage of the process, has found many opponents.

Minkowski, as early as 1892 (3) and again in 1904 (4), emphasizes the importance of alterations of the liver cells in causing jaundice and proposes his theory of "*paracholie*" or the passage of bile from the pathological liver cells directly into the perilymphatic spaces, rather than into the bile capillaries. Sterling (5) some years later arrived at the same conclusion. He was not able to observe rupture of the bile capillaries after biliary obstruction and laid emphasis on early changes in the liver cells themselves.

Ogata (6) says that before the rupture of the bile capillaries there is a dilatation due to increased pressure. He believes that a necrosis of liver cells appears in the first stages of biliary obstruction. Browicz (7), Jagie (8), Kodama (9) and Hiyeda (10) have confirmed these findings. They were unable to observe any rupture of bile capillaries in early obstructive jaundice. The last two investigators, studying liver sections from 7 to 11 hours after ligation of the ductus choledochus, could

observe only a dilatation of bile capillaries, and in some cases the formation of bile thrombi (*"Gallenthromben"*).

We must discard the early observations regarding the time of first appearance of bile in the blood stream because of faulty technique. Either the Gmelin test was used to detect the bile pigments or else the investigators tested for bile salts. We know now that the Gmelin test is the least sensitive of all bilirubin reactions, and even as yet we have no method sensitive enough to detect small traces of bile salts in blood or urine.

Eppinger (11) in 1920 stated that 24 hours after obstruction one can notice an increase in the bilirubin content of the blood. Lepehne (12) in 1921, experimenting with rabbits and employing the Van den Bergh test to detect bilirubin, observed in 3 out of 5 animals an increase of blood bilirubin from 0.4 to 6.5 units within 24 hours. Bloom (13), using dogs previously nephrectomised, noticed the presence of the indirect Van den Bergh reaction from 1 hour and 19 minutes to 4 hours after ligation of the bile ducts. Kodama (9) in 1925 observed, in rabbits and dogs, the appearance of the indirect reaction 7 hours after ligation of the ductus choledochus, the direct reaction appearing 10 hours after obstruction. More recently Bollmann, Sheard and Mann (14) employing the Keuffel and Esser spectrophotometer to detect the bilirubin showed that the increase of bilirubin in the blood begins 5 minutes after ligation of the bile ducts. They further studied the pressure within the bile ducts and they observed that it was raised from 14 mm. water pressure to 270 or 300 mm. after obstruction. In conclusion they state: "As soon as the pressure in the biliary ducts has risen to 250 or 300 mm. of water, the hepatic cells become impervious to bilirubin so that bile pigment is neither excreted into the bile capillaries nor is it absorbed from the blood by the hepatic cell."

Report of Experiments.

I. First Appearance of Bile in the Blood Stream after Biliary Obstruction.

In all of our experiments we have employed dogs previously kept in metabolic cages and known not to show the so called physiologic bilirubinuria.

The method employed to detect bilirubin in the blood and lymph has been the well known Van den Bergh test, the reactions obtained being divided into three groups:

Direct Reaction.—Instantaneous appearance of the colour reaction after the addition of the reagent, reaching its maximal intensity in from 20 to 30 seconds.

Biphasic Reaction.—(Combination of direct and indirect reaction) in which the colour reaction begins within the first 30 seconds but does not reach its maximum intensity until from 1 to 30 minutes later.

Indirect Reaction.—In which the colour reaction begins from 1 to 3 minutes or more after the addition of the reagent, requiring sometimes the addition of alcohol. The maximum intensity is reacted after a variable time and the action of alcohol is essential to produce the maximum colour.

We used according to Lepehne's technique (15) 0.25 cc. of lymph or serum to which was added 0.20 cc. of freshly prepared reagent. The quantitative estimation was made with the Bausch and Lomb microcolorimeter, with, as standard, a solution of crystallised cobalt sulfate[1] previously checked against a solution of pure bilirubin. The determinations in serum giving the indirect Van den Bergh reactions were made according to Van den Bergh's technique, and the quantitative estimations in those giving biphasic or direct reactions were made by the modification of Thannhauser and Andersen (16).

The operative technique was the same as that employed by Bloom. All the dogs were given a preparatory injection of morphine followed in 30 minutes by ether anesthesia and were kept lightly under the anesthetic until the end of the experiment.

In this series of experiments, both kidneys were removed and the ductus choledochus and cystic duct ligated through a midline abdominal incision. Samples of blood were taken every 15 minutes from the carotid artery into which a cannula had been previously introduced. When the direct Van den Bergh reaction appeared in the blood the dogs were killed with ether. At necropsy the ligatures were verified and samples of liver taken for microscopic examination.

Five experiments were performed; all gave concordant results. The following protocol gives the details in one experiment; the results of the series have been summarised in Table II.

Protocol of Experiment on Dog 8.

Female, weight 11.5 kilos. At 10 a.m. a hypodermic injection of morphine was given. At 10:30 a.m. anesthesia with ether. At 10:50 a.m. a cannula was put in the carotid artery and a sample of blood taken. At 11:15 a.m. midline abdominal incision and transperitoneal double nephrectomy. At 11:30 a.m. ligation of ductus choledochus and cystic duct. Samples of liver were taken every 15 minutes.

The experiment lasted 6 hours and 20 minutes. The dog was killed with ether and a necropsy performed. The ligatures were well placed; there was no blood in the abdominal cavity. The liver was slightly hyperemic; on section, the portal and interlobular veins were found to be full of blood; the bile ducts were full of bile. The lymph vessels looked distended and greenish in colour. The spleen was

[1] 3.915 gm. of $CoSO_4 \cdot 7H_2O$ in 100 cc. distilled water is equivalent to 0.005 mg. per cc.

full of blood but not swollen. Samples of liver were taken for microscopical sections.

At 1 p.m., that is 1 hour and 30 minutes after biliary obstruction, the blood serum gave the indirect Van den Bergh reaction, which remained until 2:15. At this time, that is 2 hours and 45 minutes after obstruction, the Van den Bergh reaction became biphasic. At 4:15 p.m. or 4 hours and 45 minutes after obstruction, the reaction became direct rising steadily until the animal was killed.

TABLE I.

Dog 8. Experimental Obstructive Jaundice in Dogs Previously Nephrectomised. Van den Bergh Reaction and Bilirubin Estimation in the Blood Serum, Withdrawn Every 15 Minutes from the Carotid Artery.

Blood.	Van den Bergh reaction			Time after obstruction	Quantitative estimation of bilirubin
Time	Direct	Biphasic	Indirect		
a.m.					*mg. per liter*
10:50	−	−	−		
11:30	Ligation of common and cystic bile ducts				
11:45	−	−	−		
12 m.	−	−	−		
p.m.					
12:15	−	−	−		
12:30	−	−	−		
12:45	−	−	−		
1	−	−	+	1 hr., 30 min.	Traces
1:15	−	−	+		"
1:30	−	−	+		"
1:50	−	−	+		"
2:15	−	+		2 hrs., 45 min.	1.50
2:35	−	+			2.00
2:55	−	+			2.50
3:15	−	+			3.50
3:35	−	+			3.75
4:15	+			4 hrs., 45 min.	4.00
4:35	+				4.50
4:55	+				5.50

In Table II we summarise the five experiments performed. We see that in the early hours after biliary obstruction in dogs, the time of appearance of bilirubin in the blood is quite constant. The indirect reaction appears during the 2nd hour after obstruction is performed and persists from 1 hour to 1 hour and 45 minutes. The biphasic reac-

tion then appears and lasts from 1 hour and 10 minutes to 2 hours and 10 minutes. Finally the direct reaction appears 4 to 5 hours after obstruction and will last as long as the obstruction persists.

The behaviour of the biphasic reaction was not always the same. Thus in Dog 7 the biphasic reaction first appeared 2 hours and 30 minutes after obstruction; in the following sample, 15 minutes later, the reaction was indirect; in the sample collected 30 minutes later it had become biphasic again.

In Fig. 1 we give the results of the quantitative estimations of bilirubin made in the five experiments. The rise in the bilirubin content of the blood, after it is first detected, is regular until the end of the

TABLE II.

Experimental Obstructive Jaundice in Nephrectomised Dogs. Summary of Experiments Showing the Time of Appearance of Van den Bergh Reaction in the Blood Serum.

Dog No.	Sex	Weight	Time of appearance of the Van den Bergh reaction in the blood serum, after total biliary obstruction		
			Indirect	Biphasic	Direct
		kg.			
3	F.	8	1 hr., 25 min.	2 hrs., 15 min.	4 hrs., 23 min.
4	F.	12.2	1 hr., 15 min.	3 hrs.	4 hrs., 10 min.
6	M.	10	1 hr., 20 min.	2 hrs., 40 min.	4 hrs.
7	F.	5.4	1 hr., 20 min.	2 hrs., 30 min.	4 hrs., 55 min.
8	F.	11.5	1 hr., 30 min.	2 hrs., 45 min.	4 hrs., 45 min.

experiment. We may add that another series of experiments now being performed indicates that the rise is continuous and slow until 40 hours after total biliary obstruction. Then a sudden rise occurs which is maintained at more or less the same level until relief of the obstruction or until death. We take from these experiments, one shown in Fig. 2, which indicates the bilirubin content in the blood during the whole period of obstruction until relieved by a biliary fistula. The blood bilirubin after relieving the biliary obstruction, behaves, as we see in this figure, in a manner exactly the reverse of that noted in early obstructive jaundice. The 3rd day after relief of the obstruction the blood bilirubin, besides diminishing in quantity, has become

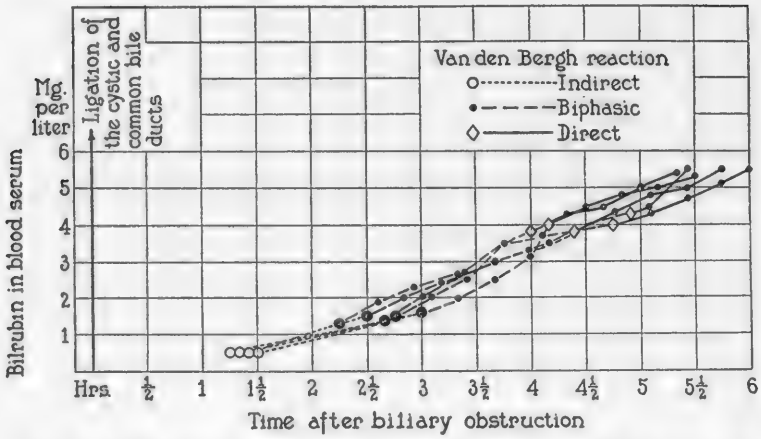

FIG. 1. Experimental obstructive jaundice in nephrectomised dogs. Curve of blood bilirubin and Van den Bergh reaction in early obstructive jaundice.

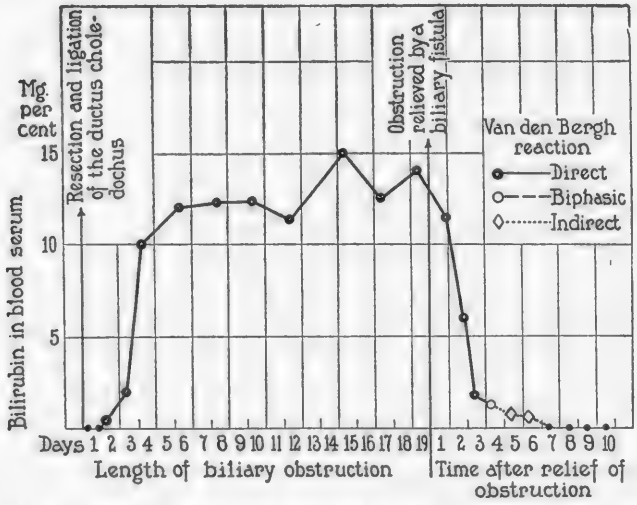

FIG. 2. Experimental obstructive jaundice in dogs. Curve of blood bilirubin during the whole period of obstruction till 7 days after relief of obstruction by a biliary fistula.

biphasic in type; on the 5th day the Van den Bergh reaction has changed into an indirect type. On the 7th day after relief of the obstruction, the bilirubin had disappeared from the blood.

II. Route Taken by the Bile to Reach the Blood Stream after Biliary Obstruction.

The question of the route taken by the bile to reach the blood stream after complete biliary obstruction has also attracted considerable attention. Many investigators have supposed the escape of bile from the liver in obstructive jaundice to be due to the activity of the lymphatic apparatus, while others uphold the view that this process is essentially an absorption of bile from the liver by the activity of the blood capillaries.

Fleischl (17) after ligating the ductus choledochus collected the lymph through a fistula made in the thoracic duct and found it charged with bile, while after 5 hours he could not detect any bile in the blood stream. Kunkel (18) and Kufferath (19) reached the same conclusion from determinations of the bile salts. Harley (20) ligated the common bile duct of two dogs, and in one in addition closed the thoracic duct. He observed in the dog with thoracic duct intact, that bile appeared in the urine in the course of a few hours after biliary obstruction, while in the dog with thoracic duct closed as long a period as 8 days elapsed before there was any such discharge. Wertheimer and Lépage (21) performed a series of experiments on dogs with a fistula of the thoracic duct and ligation of the ductus choledochus. They injected ox bile into the bile duct of the right hepatic lobe, and after a certain time could observe the presence of the cholohematin spectrum in the bile coming from the left hepatic lobe, but in these experiments they could not look for the presence of cholohematin in lymph, because, "quand le spectre de la cholohematine commençait à se montrer dans la bile du chien, la lymphe était devenue rouge." They then injected solutions of pure bilirubin under pressure and observed the appearance of the Gmelin reaction in the lymph 1 hour after the injection and its appearance in the urine 2 hours and 30 minutes after the injection. They conclude: "La verité est que les deux ordres des vaisseaux contribuent à leur resorbtion et. . . . . Le rôle des lymphatiques n'en reste pas moins important." But 1 year later (22) these same investigators performed simultaneous ligations of the ductus choledochus and thoracic duct and observed the appearance of bile in the urine. They considered the lymphatic route to be of secondary importance because they observed that bile pigment also appeared in the urine in a like manner when they ligated the ductus choledochus only. Mendel and Underhill (23) injected indigo carmine, KI, K ferrocyanide, milk and I, and milk into the common bile duct, and observed that these foreign substances appeared first in the urine

and then in the lymph with the exception of the milk-iodine mixture, which was detected 65 minutes after the injection in the lymph but not in the urine. They concluded that the hepatic capillaries are the important factor in this absorption. Whipple and King (24) ligated the common bile duct, exposed the thoracic duct and placed a ligature at its junction with the jugular vein. They detected the bile pigments in urine and lymph by the use of the Salkowski test. From their experiments they concluded that in obstructive jaundice the bile which escapes from the

TABLE III.

Dog 11. Experimental Obstructive Jaundice in Nephrectomised Dogs. Van den Bergh Reaction and Bilirubin Estimation in Samples of Lymph Withdrawn Every 20 Minutes from a Thoracic Duct Fistula, Previously Performed.

Lymph	Van den Bergh reaction			Time after obstruction	Quantitative estimation of bilirubin
Time	Direct	Biphasic	Indirect		
					mg. per liter
12:30	−	−	−		
12:40	Ligation of common and cystic bile ducts				
1:00	−	−	−		
1:20	−	−	−		
1:40	−	−	+	1 hr.	Traces
2:00	−	−	+		0.5
2:20	−	−	+		0.8
2:40	−	−	+		0.80
3:00	−	−	+		0.85
3:20	−	+		2 hrs., 40 min.	1.25
3:40	−	+			1.20
4:00	−	+			1.30
4:20	−	+			1.30
4:40	−	+			2.00
5:00	−	+			2.50
5:20	−	+		4 hrs., 40 min.	3.25
5:40	+				3.25
6:00	+				3.50
6:15	+				4.00

liver is absorbed by the blood capillaries and carried by the blood to the kidneys: "at best the lymphatic system is a secondary factor in the mechanism of jaundice." Bloom in the paper previously mentioned (13) detected bilirubin in blood and lymph by the Van den Bergh technique and observed that a positive test for bilirubin was obtained in the lymph while the blood was still pigment-free. He concluded that "during the first hours after obstruction of the common bile duct, the bile pigments are carried from the liver by the lymph stream and by it are emptied into the general circulation."

Report of Experiments.

The purpose of our second series of experiments was to study the route taken by the direct bilirubin found some hours after obstruction, to reach the blood stream.

The protocol of one experiment is given in detail; the results of the series of experiments have been summarised in Tables III and IV.

TABLE IV.

Dog 11. Experimental Obstructive Jaundice in Nephrectomised Dogs. Van den Bergh Reaction and Bilirubin Estimations in Samples of Blood Serum Withdrawn Every 20 Minutes from the Carotid Artery.

Blood	Van den Bergh reaction			Time after obstruction	Quantitative estimation of bilirubin
Time	Direct	Biphasic	Indirect		
					mg. per liter
12:20	−	−	−		
12:40	Ligation of common and cystic bile ducts				
1:05	−	−	−		
1:20	−	−	−		
1:40	−	−	−		
2:00	−	−	−		
2:20	−	−	−		
2:40	−	−	−		
3:00	−	−	−		
3:20	−	−	+	2 hrs., 40 min.	Traces
3:40	−	−	+		Traces
4:00	−	−	+		0.5
4:20	−	−	+		0.8
4:40	−	+		4 hrs.	1.00
5:00	−	+			1.30
5:20	−	+			1.50
5:40	−	+			1.50
6:00	+			5 hrs., 20 min.	2.00
6:15	+				2.00

Dog 11.—Male, weight 16 kilos. An injection of 3 cg. of morphine was given 39 minutes before ether anesthesia was begun. All the veins entering the left external jugular vein at its junction with the thoracic duct were ligated and a cannula placed in the jugular vein, at 12:15 p.m. The lymph flow was regular at a rate of 20 cc. to 30 cc. per hour. At 12:20 p.m. a cannula was inserted in the right carotid artery and a sample of blood withdrawn. At 12:32 p.m. midline incision and transperitoneal double nephrectomy. At 12:40 p.m. ligation of common and

cystic bile ducts. Samples of blood were withdrawn every 20 minutes, the lymph being examined at the same time. The indirect Van den Bergh reaction appeared first in the lymph 1 hour after the obstruction. 2 hours and 40 minutes after the obstruction the Van den Bergh reaction in the lymph became biphasic, while the indirect reaction appeared in the blood serum. 4 hours and 40 minutes after obstruction the Van den Bergh became direct in the lymph, and 40 minutes later, that is 5 hours and 20 minutes after biliary obstruction, the blood serum showed also the direct Van den Bergh reaction. The dog was killed at 6:20 p.m. with ether. At necropsy the ligatures of bile ducts were well placed. The liver appeared hyperemic. On section the bile ducts were distended with bile. The lymphatics were distended and greenish in colour. Samples of liver were taken for microscopic examinations.

TABLE V.

Experimental Obstructive Jaundice in Dogs Previously Nephrectomised. Ligation of Bile Ducts and Thoracic Duct Fistula.

Dog No.	Sex	Weight	Time of appearance of the Van den Bergh reaction after biliary obstruction in dogs with thoracic duct fistula					
			Lymph			Blood		
			Direct	Indirect	Biphasic	Direct	Indirect	Biphasic
		kg.						
11	M.	16	4 hrs., 40 min.	1 hr.	2 hrs., 40 min.	5 hrs., 20 min.	2 hrs., 40 min.	4 hrs.
14	F.	12.2	4 hrs., 20 min.	50 min.	2 hrs., 25 min.	5 hrs., 15 min.	2 hrs. 30 min.	4 hrs., 15 min.
17	F.	14.4	4 hrs., 50 min.	1 hr.	2 hrs., 50 min.	5 hrs., 45 min.	2 hrs., 55 min.	4 hrs., 30 min.

In one additional experiment the thoracic duct was ligated accidentally. We proceeded as previously, taking samples of blood every 15 minutes. In this dog, the indirect Van den Bergh first appeared in the blood 2 hours and 45 minutes after obstruction. The biphasic reaction appeared 4 hours and 30 minutes and the direct reaction 5 hours and 30 minutes after obstruction. The Van den Bergh reaction in this case behaved as it did in the experiments with thoracic duct fistula.

We summarise in Table V the results of our three experiments, which are in close agreement.

We see from these experiments that after complete biliary obstruc-

tion associated with a thoracic duct fistula, the bile appears first in the thoracic duct before it is detectable in the blood, but that 1 hour later it is present in the blood, not only when the lymph is being drained from the thoracic duct, but even when the duct has been ligated.

Microscopic Examinations of the Liver of These Dogs, 5 to 6 Hours after Total Biliary Obstruction.

It is generally agreed that rupture of bile capillaries appears only in the late stages of biliary obstruction. We made microscopical examinations of the liver of the dogs reported in our experiments. Hematoxylin and eosin sections showed no atrophy or necrosis of the liver cells which appeared quite normal. The bile ducts were not noticeably distended and no bile thrombi were observed. The Kupffer cells in the liver of Dog 8 (whose protocol has been given) contained a moderate amount of brown pigment which could be stained for iron. No such pigment was found in any of the others. Frozen sections stained with Scharlach R revealed only a slight amount of finely divided fat in the liver cells. The cells lining the smaller bile ducts consistently contained fat droplets of various size. Sections were fixed and stained by the method described by Vance (25) for the demonstration of bile canaliculi. The canaliculi were well stained, prominent and slightly distended but the most careful search failed to reveal the rupture of any of them into adjacent lymph spaces.

DISCUSSION.

Taking first in consideration the behaviour of the Van den Bergh reaction in early obstructive jaundice, we see from our experiments that the indirect reaction appears first, then the biphasic and some hours later the direct reaction, which remains during the whole period of obstruction. In spite of the many investigations carried out concerning the nature of the Van den Bergh reaction, it still remains an unsettled problem. Nevertheless from McNee's (26) and Van den Bergh's (27) researches corroborated by Andrews' important work (28), it is clear that the bilirubin normally present in the circulating blood gives only the indirect reaction, and that on passing through the liver cell during excretion, it is changed in some as yet undeter-

mined manner into a form which gives the direct reaction.[2] What is the significance of the indirect reaction which uniformly appears in the plasma of this animal soon after obstruction, and lasts a short time? It seems to us that a possible explanation for this phenomenon would be that as a consequence of the biliary obstruction a nervous reflex may occur which would lead to sudden paralysis of the function of the liver cells and as a result the circulating bilirubin would remain in the blood stream. This would be analogous to the temporary reflex paralysis of the excretory function of the kidney after ligation of the ureters, which is a well known phenomenon. As the process of bilirubin formation goes on, the bilirubin would soon reach a level sufficient to give a positive test with the diazo reagent. This reflex phenomenon would not last very long. Some hours after obstruction the liver cells would resume their function and the bilirubin giving the indirect reaction would be changed by them into bilirubin giving the direct reaction. We are at present engaged in a study of this possibility.

Another difficult aspect of the problem now presents itself. If in the first hours of obstructive jaundice there is no rupture of bile canaliculi, by what process does the direct reacting bile appear in the blood? In short what is the intimate mechanism of early obstructive jaundice? This is a field in which suggestions only can be made. Must we return to Minkowski's theory as offering the only plausible explanation? The double polarization of the liver cells has been known since Claude Bernard's memorable discovery of the glycogenic function of the liver (29). We know that the epithelial liver cell can function in two different ways: as a gland of internal secretion expelling its products into the blood stream and as a gland of external secretion excreting bile into the bile canaliculi. But it is extremely difficult to comprehend by what mechanism the excretion of bile can shift from an external to an internal secretion.

A close examination of the microscopic sections of the liver in early obstructive jaundice, stained by Vance's technique, shows the bile canaliculi dilated and extending between the liver cells in small distended pouches, the blind end of these lying in contact with the

[2] In the dog, bilirubin is present in the plasma normally in amounts too small to be detected by the Van den Bergh test.

pericapillary spaces. It would be quite easy to believe that bile might diffuse from these thin walled pouches into the tissue spaces without any actual rupture of the bile canaliculi. This diffusion would be favored by the mounting pressure inside the bile ducts, which rises rapidly according to Bollmann, Sheard and Mann (14).

Regarding the second question, that of the route taken by the bile to reach the blood, since, as we have seen in the thoracic duct fistula experiments, direct reacting bilirubin soon finds its way into the perivascular lymph spaces which surround the hepatic capillaries, bile may diffuse into these capillaries and thus directly enter the blood stream, as we have observed in our experiments.

We think our experiments explain the contradictory results of other investigators concerning the route by which bilirubin reaches the blood in obstructive jaundice. We feel justified in concluding as Wertheimer and Lépage did after their first observations: "La verité est que les deux ordres des vaisseaux contribuent à leur resorbtion et . . . . Le rôle des lymphatiques n'en reste pas moins important."

CONCLUSIONS.

1. After experimental ligation of the bile ducts in dogs, two distinct processes are clearly manifested: first, the accumulation of the normally circulating bilirubin in the blood with its characteristic indirect Van den Bergh reaction for a period of several hours, and second, the subsequent appearance of the bile bilirubin giving the direct Van den Bergh reaction. It is possible that the first process may be due to a temporary reflex inhibition of the function of the liver cells due to ligation of the duct and comparable to the same phenomenon which usually occurs in the kidney when the ureter is ligated. The second process begins before any rupture of the bile capillaries is visible. Liver sections made 6 to 7 hours after obstruction show these bile capillaries dilated and extending between the liver cells in small distended pouches the blind end of these lying in contact with the pericapillary spaces. It is possible that bile may diffuse from these thin walled pouches into the perivascular lymph spaces, this diffusion being favored by the mounting pressure inside the bile ducts.

2. In early obstructive jaundice bile first appears in the lymph, but exclusion of the thoracic duct from the circulation by drainage causes

a delay of only a few hours in the appearance of bile bilirubin in the blood stream. We must therefore conclude that after biliary obstruction bile enters the circulation both by way of the blood capillaries and the lymphatics, although the latter route is the more important.

We wish to express our thanks to Dr. Arnold R. Rich for his constant assistance and many valuable suggestions.

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PHOTOSENSITIZATION OF ANIMALS AFTER THE INGESTION OF BUCKWHEAT.

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It has been known for a long time that certain animals, following the ingestion of various substances, are rendered sensitive to certain types of radiant energy. The present study covers biologic and physical observations on the degree of sensitization, the character of the sensitizing light and the nature of the photodynamic substance of buckwheat. White rabbits, mice, rats, goats, swine, dogs and varicolored guinea pigs were studied. Sunlight, carbon arc lamp (Efka or Hoffman) with Conradty-Norris vacuum carbon electrodes and quartz mercury vapor arcs (Victor X-Ray Corporation), both with and without various filters, were employed for irradiation. All of these sources contain infra-red, visible and ultra-violet radiations.

RÉSUMÉ OF LITERATURE.

Considerable literature, especially European, has appeared on the subject of diseases in animals and man brought about by optical sensitization. Somewhat detailed accounts of the contributions relative to diseases due to exogenous sensitization are to be found in the brochures by Hausmann and by Mayer.

Years ago European stockmen found that in certain animals which had ingested buckwheat (plant or seed), erythema, itching, edema and convulsions developed and, in many cases, paralysis and death, on exposure to out-of-door sunshine. However, untoward symptoms did not arise if the animals were kept indoors or in partial darkness and they recovered from the sensitization induced by eating certain plants if they were removed from the light early in the onset of the symptoms. Likewise it was found that the non-pigmented areas of the body were affected readily, whereas the pigmented areas were kept intact. A relatively high degree of intensity of light appears to be a factor in all such diseases as so called buckwheat poisoning (fagopyrismus). Buckwheat causes sensitization to light

which may persist for a few weeks after the last ingestion of this substance provided the animals are kept in a dark place or if cloudy weather intervenes. This is true also after the experimental ingestion of hematoporphyrine.

Finsen observed that such photosensitizations were due to an optically active substance. He studied the influence of daylight on the course of smallpox, in which it would appear that the short or chemical rays must be avoided. He pointed out the similarity between this affection in man and buckwheat poisoning of animals.

At first it was thought that the etiology of fagopyrismus was to be found in secondary organisms, which were believed to live on the plants as parasites, and that these suffered certain changes under the influence of sunlight. Von Tappeiner, however, found in his experiments on photodynamic substances that sickness due to buckwheat could be induced by the removal of fluorescent material from the plant after its exposure to light. Merian arrived at the same conclusions as previous investigators but added the suggestion that the sensitization produced might be due to the longer waves of the visible portion of sunlight. The idea is prevalent in the literature that, since herbivorous animals in their ingestion of food take in considerable quantities of photodynamically active substances, especially chlorophyll, the frequent appearance of diseases due to exogenous sensitization should be expected. Apparently, however, these substances are not taken up by the animal organism in photodynamically active form or they are rendered inactive by some process in the body.

Methods of Experiments.

Feedings.—The goats, rabbits and guinea pigs were fed freshly cut buckwheat and dry seed. The swine received macerated green plant, with milk and bread, in the relative ratio of 2:1. The dogs were fed macerated green buckwheat and chopped meat in equal parts. The rats and mice were fed dry seeds only.

Irradiation.—All animals were kept in cages or stalls away from direct light. During exposure they were placed in a wire mesh cage with an open top. In the investigation of the effects of selective solar irradiation a metallic box was used, over the top of which windows of different absorptive filters were placed as desired. The whole apparatus could be set at angles to bring all animals under the direct rays of the sun.

The Efka carbon arc was operated on 110 volts alternating current and carried 10 amperes. By reason of its construction (with a spherical reflector so angled as to concentrate much of the light and heat from the lamp at a point directly beneath the arc), it was necessary to place the animals in a metallic box built so as to avoid shadows when it was placed about 30 cm. directly in front of the arc proper. Glass filters were fitted in the apertures at the side and top of the box when desired.

An air-cooled quartz mercury vapor lamp was operated at 70 volts and at a distance of 50 cm. from an open topped polished metal box, in which were placed the animals to be irradiated.

The dogs, swine and goats were placed in open areas or fields during irradiation, thus bringing them under a natural environment and permitting slight reactions to be noted.

Observations on Animals after Irradiation.

Sunlight.—After 4 days of the feeding of fresh buckwheat, guinea pigs became photosensitive to direct sunlight. These were the earliest reactions noted. The symptoms, in the order of appearance, were: shaking of the head, excitation, squeaking, scratching of the ears and intense agitation. They attempted to protect themselves by huddling and crawling under each other. The degree of photosensitization increased with continued feeding of buckwheat until, at the end of a week, an exposure of 20 minutes to direct sunlight caused symptoms similar to those in anaphylaxis. The animals were calmed only with complete darkness.

The swine were next in susceptibility. They exhibited marked symptoms of photosensitization on the 10th day of feeding buckwheat. However, the symptoms were not the same as those manifested by the guinea pigs. The reactions noted were: itching, weakness and extensive urticaria followed by sloughing. The first reactions were observed after about 2 hours of direct solar irradiation.

The goats manifested only itching and weakness after 14 days of feeding.

Rabbits, dogs and white rats did not manifest symptoms of photosensitization following direct solar irradiation. The rabbits were exposed to sunlight on every clear day during the feeding of buckwheat for a period of 30 days. No evidences of sensitization were observed even after exposure for 5 hours to direct sunlight. Dogs kept on a diet consisting of 50 per cent buckwheat for 3 weeks were exposed daily to direct sunlight without visible reactions or discomfort, although the periods of exposure were increased to 6 hours. The white rats did not exhibit symptoms of photosensitization.

Quartz Mercury Vapor Lamp.—Irradiation by a quartz mercury lamp did not produce symptoms of sensitization to light in animals fed on buckwheat (plant or seeds). If anything, this type of irradiation appeared to build an additional resistance to photosensitization, probably because an increase in pigmentation was produced.

Carbon Arc.—With the carbon arc, the biologic effects were similar to those produced under direct sunlight but were much less severe.

The Region of Photosensitizing Action.

In order to test whether or not there were any differences in physiologic reactions under various portions of the visible solar spectrum, amber and blue glass filters were used. Light-tight boxes were made and fitted with covers carrying the selected filters. Sensitized animals (guinea pigs) were placed beneath these filters and under direct solar irradiation. Animals under the amber glass (Pittsburgh 48, hammered cathedral) evidenced the same excitability, irritation, itching and scratching as shown by other (or the same) sensitized animals under full sunlight. Animals under blue glass (Pittsburgh 351 or 56, hammered cathedral) remained quiescent and behaved for the most part as did animals having no buckwheat in their diet.

Spectroscopic curves showing the percentages of transmission of light of various wave-lengths by the filters used are shown in Fig. 1. Since the blue filters transmit the region of short wave-lengths (400 to 500 millimicrons) to a maximal amount and since animals fed on buckwheat do not show reaction under such filters, it is apparent that the blue-violet portion of sunlight, together with the near ultra-violet portion also transmitted, is not the photosensitizing region.

Hausmann (1911, 1914) demonstrated that hematoporphyrine has a photodynamic action. It was shown by Freund that eruption in hydroa is produced by wave-lengths from 325 to 396 millimicrons. However, it would appear from the literature that, in experimental sensitization with hematoporphyrine, it is not the invisible but rather the visible portion of the spectrum that is active, namely that in the region of 500 millimicrons.

Certain observers have felt that, by reason of the close parallelism between the chemical and physiologic properties of hemoglobin and chlorophyll and their derivatives (Palladin), such as phylloporphyrine and hematoporphyrine, hematoporphyrine was possibly the photosensitizing agent in the case of buckwheat disease. If the statement can be accepted that the region of 500 millimicrons is the photodynamic portion of the spectrum in the case of hematoporphyrine, then this substance is ruled out of consideration in the present experi-

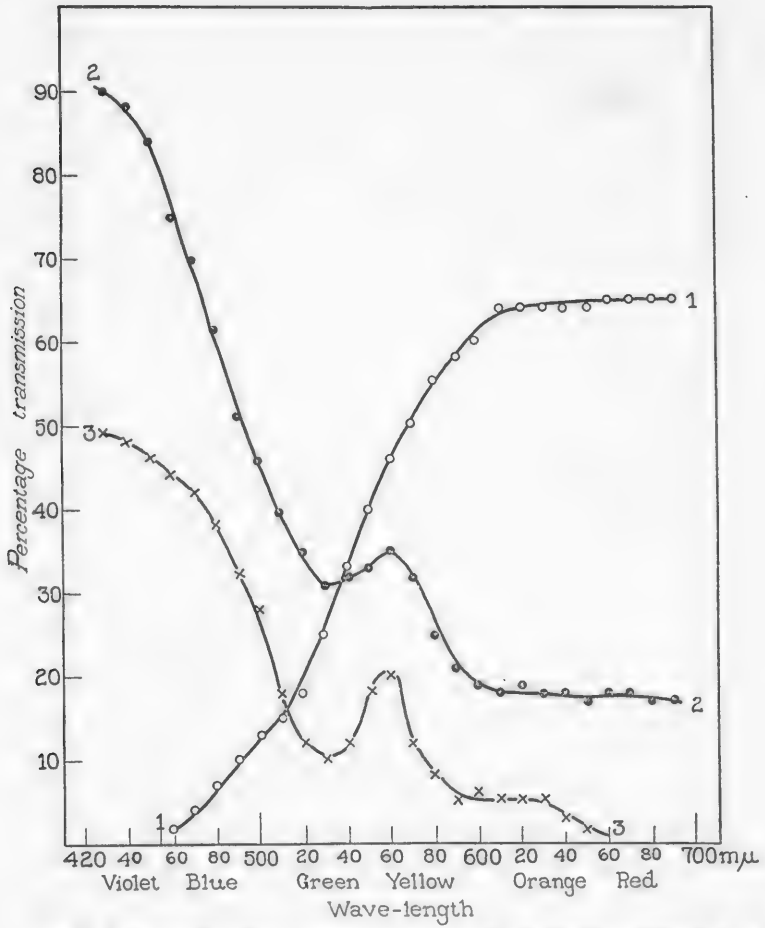

FIG. 1. Spectrophotometric determinations of the percentage transmission of the various filters used. Curve 1, Pittsburgh 48 amber; Curve 2, Pittsburgh 56 blue; Curve 3, Pittsburgh 351 blue glass.

ments for the reason that the energies transmitted by the filters used (Fig. 1) in the region of 500 millimicrons are comparable, yet photodynamic action occurs under the amber filter but not under the blue.

Neither can the difference in physiologic action of the radiant energy transmitted by the different filters be attributed to infra-red radiation. Fig. 2 shows the percentage transmission of the amber and blue filters for wave-lengths of from 750 to 4250 millimicrons. These curves are comparable in all respects; if anything, the blue filter, under which no sensitization occurs, transmits about 10 per cent

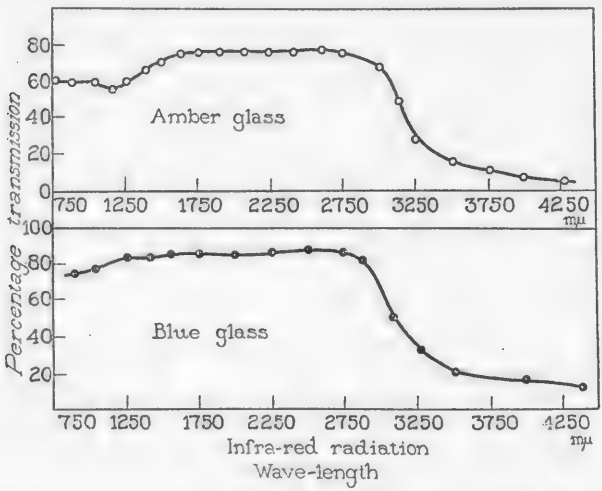

FIG. 2. Curves showing the percentage transmission of amber and blue glass filters for infra-red radiations covering the range of 750 to 4250 millimicrons.

(on the average) more infra-red radiation than does the amber filter (Sheard).

From the character of the physiologic and pathologic reactions produced under various filters and from a consideration of the percentages of transmission of solar energy in the visible spectrum, we conclude that the region of photosensitization lies between 580 millimicrons and the red end of the spectrum.

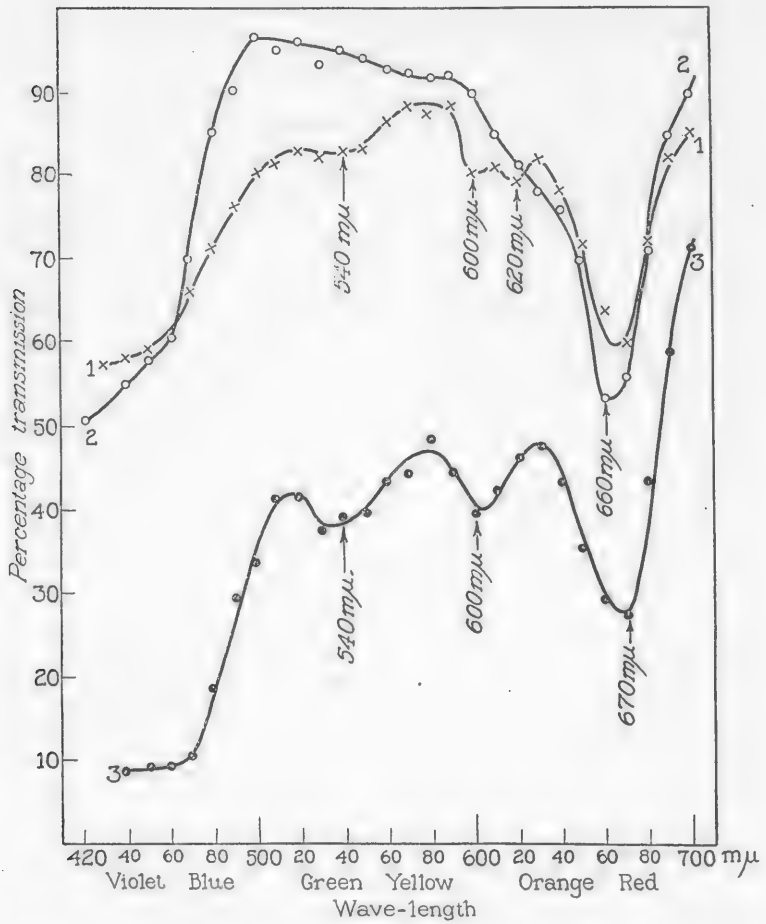

FIG. 3. Spectrophotometric determinations of the percentage transmission of alcoholic extracts of plants. 5 cm. tubes were used in all cases. Curve 1, leaves of buckwheat; Curve 2, grass; Curve 3, stalks of buckwheat.

Spectrophotometric Determinations of Chlorophyll.

From our experience with ethyl alcoholic solutions of chlorophyll (of sufficient dilution) from various sources we believe that such solutions will show two absorption zones, namely, from the limit of the violet end of the visible spectrum to about 500 millimicrons and from 630 millimicrons to approximately 680 millimicrons with a point of maximal absorption at from 660 to 670 millimicrons. That is to say, there is marked absorption in living leaves and in alcoholic solutions of chlorophyll (ethyl chlorophyllide) in the extreme violet and remote red ends of the spectrum. Besides these absorption bands or zones, certain other alcoholic solutions of chlorophyll of equal concentration (as judged by the degree of absorption in the violet and red regions) exhibit additional bands. Curve 2 of Fig. 3 (alcoholic extract from grass) and Curve 1 of the same figure (alcoholic extract of buckwheat leaves) exhibit practical equality of absorption in the regions 430 to 490 millimicrons and 630 to 690 millimicrons. The buckwheat extract, however, exhibits two additional bands with maximal absorption zones at about 540 and 600 millimicrons, as is shown definitely in Curve 3 of Fig. 3 (alcoholic extract of stems of buckwheat). These data may indicate that there are two forms of chlorophyll, such as chlorophyll *a*, and chlorophyll *b*, suggested by Willstätter and Stoll or that there are derivatives of chlorophyll present in certain plants (such as buckwheat) that are not found in other vegetation, such as grass.

The Spectrophotometric Determinations of Blood Serums of Sensitized Animals.

Several attempts were made to obtain satisfactory spectroscopic and spectrophotometric tests on the blood serums of normal and sensitized guinea pigs. Difficulties connected with the prevention of hemolysis of considerable degree, however, did not warrant the drawing of conclusions with respect to the differences in character of the spectrophotometric data, since hemolysis (as evidenced by absorption bands with a maximum at practically 540 and 580 millimicrons) would also cause an increase in spectrophotometric absorption in the blue-violet zone (490 to 430 millimicrons).

The same difficulties likewise existed in general in the serums from swine. In one case, however, serums from a normal and a sensitized pig were obtained relatively free from hemolysis. Curve 1, Fig. 4, shows the spectrophotometric results on the blood serum of a normal pig and Curve 2 contains similar data on the animal photosensitized

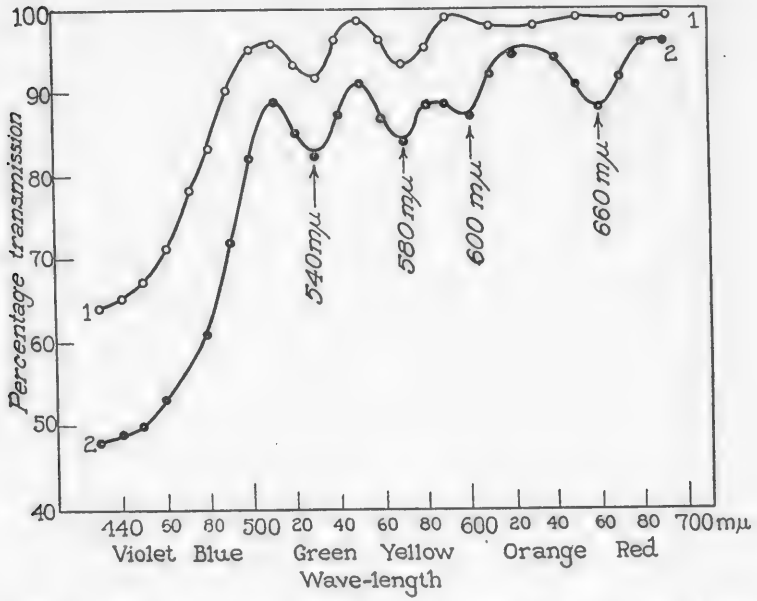

FIG. 4. Spectrophotometric curves showing percentage transmission of light by the blood serum of a normal pig (Curve 1) and by the blood serum of a photosensitized pig (Curve 2).

by buckwheat. Both curves demonstrate the presence of small but practically equal hemolysis, as shown by the absorption bands at 540 and 580 millimicrons. Curve 2, however, shows two additional absorption zones with a maximum at practically 600 and 660 millimicrons, with increased absorption in the blue-violet region (430 to 490 millimicrons).

Pathologic Data.

The anatomic changes noted in cases of buckwheat disease vary according to the length and intensity of the exposure of the sensitized animals. After a short exposure (20 minutes) of a sensitized animal to sunlight, the most noticeable change observed microscopically is hyperemia of the skin, and in most cases accompanying edema. In severe reactions the changes already noted occur and blebs and ulcers may also form. Later, crusts may be formed on the surface. Hemorrhage into the areolar connective tissue may also occur.

Fatal reactions generally demonstrated the anatomic changes mentioned, but there may also be petechial hemorrhage of the lungs, brain, liver, stomach and kidneys. Parenchymatous degeneration is usually found. The most striking feature at the postmortem examination of a photosensitized animal which has died from exposure is the lack of any marked pathologic change. The lesions found suggest the presence of profound toxemia.

In animals sensitized with buckwheat, and exposed to irradiation for a few minutes at a time and over a sufficient number of days, pigmentation involving the basal layer of the skin develops in unpigmented areas. This pigment may be distributed also in the adjacent squamous epithelium and even in the fibrous connective tissue of the deeper layers of the skin. Edema and hyperemia are present also in the skin of those animals having chronic experimental buckwheat disease. The outer layers of the skin are thickened and usually there is free desquamation. Some observers have described hypertrophy of the cartilage of the ears as associated with the hyperkeratosis but it has not been found in our series of animals. In none of the animals observed (not even those exposed to daily periods of irradiation over a period of 6 months) has any lesion developed which we felt suggested a malignant neoplasm.

DISCUSSION.

General.—Urticaria has been noted in domesticated animals pasturing on certain plants. The malady is especially noticeable when white swine pasture on rape and certain clovers. Urticaria on the non-pigmented areas of skin of Holstein and Guernsey cattle is frequently observed. If the animals are kept in darkened stables the

disease soon disappears. Stockmen have attributed the disease to certain insects, dew, nettle rash and plant secretions. Without doubt this affection appears in susceptible animals following the ingestion of certain plants with subsequent exposure to unfiltered sunlight. It may be that many plants are capable of producing this condition and that in ruminating animals the active compound undergoes chemical changes during the long process of fermentation and digestion and is thereby rendered harmless. Therefore, it is possible that photodynamic reactions occur only when large quantities of these plants are ingested and the entire organism becomes saturated.

Pathology.—The anatomic changes noted in sensitized animals that were exposed for brief periods to irradiation are about the same as those found in normal animals after prolonged exposures. Photosensitization therefore accelerates anatomic changes. Following a fatal dosage of light, the anatomic changes (petechial hemorrhages and parenchymatous degeneration) suggest that death is due to profound toxemia which kills the organism before extensive anatomic changes develop. In animals experiencing severe reaction there is, apparently, marked increase in the coagulability of the blood. It is difficult to draw blood from such animals. Postmortem examinations of photosensitized animals, whether killed by the effects of irradiation or by other means, almost always revealed the blood in firm clots in the largest vessels.

The Region of Photosensitizing Action.—Photodynamic action is brought about by the portion of radiant energy which is absorbed by the sensitizing substance. The majority of photodynamic actions which can be attributed to pigment are caused by the visible portions of the spectrum. It follows, therefore, that photodynamic effects are due ordinarily to the longer wave-lengths of visible light. The laws of photochemical reactions apply to photodynamic actions. Temperature has but little effect. The reactions which have been described in this paper were not influenced by extremes of external temperature, for they occurred about as readily on a cool day as on a hot day.

The ultra-violet portion of sunlight, carbon arcs or quartz mercury vapor lamps does not appear to be effective in photosensitization following the ingestion of buckwheat. It is claimed that the presence of hematoporphyrine (which is a derivative of hemoglobin most closely

related chemically to phylloporphyrine, a derivative of chlorophyll) brings about drastic reactions, (under irradiation of wave-lengths from 260 to 400 millimicrons), of the same general character as those produced by buckwheat. Various authorities claim that hydroa æstivale, xeroderma pigmentosum, seaman's skin and smallpox are either caused by or unfavorably influenced by ultra-violet light or the chemically active rays of sunlight (Mayer, pages 167–169). In our experiments, irradiation with ultra-violet light from a quartz mercury vapor lamp did not produce sensitization, but on the contrary built up a resistance to the untoward effects ordinarily produced when buckwheat-sensitized animals were exposed to the longer wave-lengths of sunlight. These observations lead us to believe that (1) exogenous sensitization to light, as induced by buckwheat, is not due to ultra-violet rays, (2) the effect of ultra-violet irradiation in such cases is beneficial, since the development of pigment acts as a protective agent, thereby changing the skin of the animal from a light color to a dark color and (3) hematoporphyrine, in all probability, is not the active substance.

Spectrophotometric Studies of Chlorophyll and of Blood Serums of Sensitized and Non-Sensitized Animals.—The curves of Fig. 4 we believe demonstrate the presence of chlorophyll or its derivatives in the blood of animals photosensitized by buckwheat. Attempts were made to sensitize dogs to buckwheat but were found to be unsuccessful after a period of daily feeding of buckwheat for over 3 weeks. There is evidence to show that, in all probability, none of the chlorophyll was taken into the blood through absorption by the walls of the digestive tracts of the dogs used in these experiments. Bollman, Sheard and Mann have shown that bilirubin was not formed from chlorophyll under conditions which allowed the formation of bilirubin from hemoglobin. Herbivora excrete chlorophyll in the bile during periods when their rations are rich in chlorophyll. According to Broun, McMaster and Rous, sheep show definitely that chlorophyll may be altered in the body, since a greenish pigment, cholehematin, is produced, when the animal is on green food. This pigment is also excreted in the bile. When cholehematin is administered to dogs it is excreted as such in the bile.

The Nature of the Photodynamic Substance.

According to von Tappeiner, the active principle in photosensitization brought on by buckwheat is an alcohol-soluble fluorescent substance of a nature similar to that of chlorophyllan as described by Hoppe-Seyler. According to Busck, there exists a fluorescent material, fluorophyll, which can be obtained from buckwheat. Alcoholic extracts of buckwheat are said to be inactive in both irradiated and non-irradiated animals. According to Öhmke, such extracts have true fluorescence and the residue obtained from evaporation in a vacuum, when given by mouth to white mice, caused signs of paralysis and even death after the exposure of the animals to light. Von Tappeiner states that the colored material which incites photosensitization is soluble in alcohol.

Fluoresence is a physical property commonly attributed to photodynamic substances or associated with photosensitization. That is, substances producing photodynamic action are able to absorb light and usually fluoresce; the degree of photodynamic action, however, is not proportional to the degree of fluorescence. Only that region of the spectrum in which energy is absorbed by the fluorescent substance (or substances) is effective. It is thought that the presence of a fluorescent or photodynamic substance causes tissues to become susceptible to destruction by the energy contained in wave-lengths of light that would be ineffective otherwise, namely, the longer waves of light, which are able to penetrate deeper into the tissues.

We doubt the necessity of fluorescent action or the need of a fluorescing substance at any point of the photosensitizing process if the reactions produced by the ingestion of buckwheat are to be attributed to the chlorophyll constituent *per se* rather than to some other substance present in buckwheat or to some substance formed in the blood after the ingestion of buckwheat. Our reasons are:

1. Alcoholic and other extracts of grass and buckwheat (presumably largely chlorophyll) give an orange to reddish fluorescence when exposed to irradiation by the violet-blue end of the spectrum. So far as we know, grass is not toxic, whereas buckwheat is.

2. Fluorescence is a phenomenon in which various substances absorb radiation of a certain wave-length (or region) and reemit it as

radiation of a longer wave-length. Fluorescence of chlorophyll in the longer wave-length portion of the spectrum is caused by the absorption of energy below a wave-length of 500 millimicrons. However, exposure of animals after the ingestion of buckwheat to the shorter waves of the visible spectrum does not produce photosensitization. Hence it would appear logical to conclude that the fluorescence of chlorophyll *per se* is not involved in the sensitization brought about by buckwheat.

We venture the suggestion that the photodynamic substance may be phylloporphyrine. The researches of Schunk and Marchlewski have contributed much to an understanding of the chemical character of chlorophyll. The action of hydrochloric acid on a solution of chlorophyll produces, first, chlorophyllan, then phylloxanthin and finally phyllocyanin. Phylloporphyrine is obtained by treating phyllocyanin with strong alkalies. We believe such a substance may possibly be present in the blood of sensitized animals, the hydrochloric acid of the stomach, by its action on chlorophyll, producing chlorophyllan which, on absorption into the blood with an alkalinity represented by pH 7.4, produces phylloporphyrine. Phylloporphyrine is of interest because of its close relationship to hematoporphyrine. The spectrums of the two substances in various solvents are similar. The spectrophotometric curve of the serum of blood from photosensitive animals corresponds in many particulars with that of an alcoholic solution of phylloporphyrine.

It is possible, also, that cholehematin, or its precursor, may be the photodynamic substance. We have a series of experiments under way with reference to this possibility.

SUMMARY.

The chief points presented in this paper are:

1. Following the ingestion of buckwheat (plant or seed) varicolored guinea pigs, white swine and goats exhibited symptoms of photosensitization, the degree of sensitization being in the order given.

2. Rabbits, dogs, white mice and rats did not manifest symptoms of photosensitization.

3. The symptoms and reactions were: agitation, itching, scratching

of the ears, weakness, urticaria with sloughing and symptoms similar to those in anaphylaxis.

4. Microscopic examinations showed the lack of marked pathologic change. The lesions, such as petechial hemorrhage of the lungs, brain, liver, stomach and kidneys, suggest that profound toxemia has been present.

5. Lesions were not found which appeared to be suggestive of malignant neoplasms.

6. Irradiation by a quartz mercury vapor lamp apparently develops a resistance to photosensitization, probably because of increased pigmentation induced by ultra-violet light.

7. From the nature of the physiologic and pathologic reactions produced under various filters and from a consideration of the percentages of transmission of solar energy in the visible spectrum, it would seem that the region of photosensitization lies between 580 millimicrons (yellow) and the red end of the spectrum. This conclusion, moreover, is substantiated by the fact that irradiation by a quartz mercury vapor lamp (which radiates no energy in the visible spectrum at a wave-length greater than 579 millimicrons) produces no symptoms or reactions.

8. Spectrophotometric determinations of alcoholic extracts of grass (non-toxic) and of buckwheat (toxic) show the presence of two additional bands in the absorption spectrum of buckwheat with maxima at about 540 and 600 millimicrons, respectively, together with the common absorption zones at 430 to 490 millimicrons and 630 to 690 millimicrons.

9. Spectrophotometric determinations of blood serums of sensitized animals show, besides the usual absorption bands peculiar to oxyhemoglobin (with maxima at 540 and 580 millimicrons respectively), two zones with maxima at 600 and 660 millimicrons respectively.

10. The fluorescence of chlorophyll *per se*, as suggested by previous investigators, is not, in all probability, the cause of the sensitization induced by buckwheat.

11. Hematoporphyrine is not the photodynamic substance in all probability.

12. Phylloporphyrine may be the photodynamic substance. In this regard, also, the possibility of cholehematin is not to be ruled out.

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